Spontaneous and evoked vesicle cycling at a presynaptic terminal

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Abstract

The presynaptic vesicle cycle is crucial for chemical neurotransmission. In response to an action potential, neurotransmitter-filled vesicles are exocytosed from the pre-synaptic terminal and activate specific receptors at the post-synaptic membrane. To sustain neuronal activity, vesicles are then retrieved by endocytosis and refilled with neurotransmitters for further rounds of release. Neurotransmitter release can also occur spontaneously in the absence of action potentials. The current assumption is that both types of release use the same set of vesicles. To check this hypothesis, I developed a genetically encoded probe, biosyn, to specifically label pre-synaptic vesicles in living neurons. It consists of a fusion protein between VAMP2 and a biotin acceptor peptide (BAP). Co-transfection of biosyn with BirA, an enzyme that specifically recognizes the BAP sequence, results in the biotinylation of VAMP2. The subsequent addition of fluorescently-labeled streptavidin enables the visualization of newly exocytosed vesicles. I show in cultured hippocampal neurons that this approach successfully tagged synaptic vesicles that had undergone a cycle of exocytosis and re-endocytosis without affecting synaptic function. Using different stimulation protocols, I then revealed the existence of two distinct pools of vesicles that recycle independently and with different kinetics at the presynaptic terminal. One pool is mobilized in response to neuronal activity whereas the other fuses spontaneously with the plasma membrane. I also report that the resting pool of vesicles, which cannot be mobilized by neuronal activity, is the source of spontaneous vesicle release. Finally, I describe a switch in the modes of synaptic vesicle fusion during development. Young neurons (before synapse formation) showed high levels of spontaneous, but not activity-dependent, cycling, whereas in mature neurons activity-dependent release was predominant over spontaneous fusion. These findings suggest that the spontaneous and activity-dependent modes of release may play different roles in synaptic function and during neuronal development.

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Abbreviations

ADBE	activity-dependent bulk endocytosis
AP	action potential
APV	2-amino-5-phosphonovaleric acid
Ca^{2+}	calcium
Baf	bafilomycin
BAP	biotin acceptor peptide
CAZ	cytomatrix of the active zone
CHO cells	Chinese Hamster Ovary cells
CME	clathrin mediated endocytosis
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DIV	days *in vitro*
EGFP	enhanced green fluorescent protein
EM	electron microscopy
ER	endoplasmic reticulum
FM4-6	*N*(3triethylammoniumpropyl)4(6(4diethylamino)phenyl)hexatrienyl) Pyridinium dibromide
HBS	Hepes Buffered Saline
Hc	heavy chain
High K^+	high potassium
Lc	light chain
PPVs	Plasmalemmal Precursor Vesicles
PSD	postsynaptic density
PSP	postsynaptic potential
PTVs	piccolo transport vesicles
RP	reserve pool
RRP	readily releasable pool
SNARE	soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor
strep488	streptavidin-AlexaFluor488
sypHy	synaptophysin-pHluorin
Syt1	synaptotagmin 1
TdT	Tandem-dimer Tomato
TeNT	Tetanus neurotoxin
TTX	tetrodotoxin
VAMP2	vesicle-associated membrane protein 2

CHAPTER I

Introduction

1.1- History of neurotransmission in the central nervous system

A central nervous system, consisting of brain and nerves responsible for intelligence, emotion and movements, was first postulated by the Alexandrian School of thought 300 years BC. A few centuries later, the Greek philosopher Galen ascribed a physiological function to the brain as a substance "*spiritus*" that would arise from the brain and go through the nerves to a target, such as the muscle, inducing its contraction. In the seventeenth century, Descartes gave rise to a more mechanical explanation linking the anatomy of the nervous system with the behaviour of the human body. He postulated that the internal part of the brain was composed of fine nervous fibres separated by small spaces that form a network. These fine fibres would then extend to the external part of the brain and form the nerves connecting different parts of the body. Descartes described the "*spiritus*" as being small particles in rapid movement rather than a fluid present in the brain and nerves. At the time, the nerves were considered as tubes through which the "*spiritus*" would be targeted to different parts of the body.

In the early nineteenth century, the description of the cell as a single unit gave birth to the cellular theory. In 1855 this theory was strengthened when cells were identified as being essential components for living organisms. In the mid-nineteenth century, important technical innovations in cyto-histology permitted the publication of the first microscopic image of the nerve cell's soma. Following on this work, in the late nineteenth century, Santiago Ramón y Cajal put forward the theory that the brain is composed of autonomous nerve cells. This discovery is the basis of the Neuron Doctrine, which is in accordance with the "cellular theory" as opposed to the "reticular theory" supported by Gerlach and Golgi. The latter theory sustained the idea that the central nerve cells were organised in continuous neural fibres. Following the collapse of the "reticular theory", the nerve cell gained its final terminology as a neuron and its processes were named dendrites and axons.

With the “Law of Dynamic Polarity”, Cajal hypothesised a functional role to the highly polarised morphology of the neuron within a circuit. The neuron would receive information through the dendrites and transmit the signal through the axon to another neuron. This implies that neurons have to establish appropriate connections to form a functional circuit. The concept of the synapse was first suggested by Cajal in a review he published in 1889, where he mentioned “*that nervous propagation is verified by contacts at the level of certain apparatus*”. Influenced by Cajal’s work, Sherrington defined in 1897 a physiological role of the synapse within the nervous system, as representing a single unit formed of the tip of the axon barely touching the dendrites. This specialised apparatus would transform the nervous pulse as it passes from one neuron to another.

For a century, synaptic transmission was considered to be electrical until Elliot and Langley, with their work on the peripheral nervous system, proposed it to be chemically-mediated. Thereafter, they hypothesised that the action potential would propagate from one cell to another through the synapse via the release of chemical substances named neurotransmitters. A year later, they discovered the existence of synaptic receptors, further confirming their ideas (Langley, 1905). The transfer of information by neurotransmitters was well accepted in the peripheral nervous system. In 1950, progress in electron microscopy permitted the confirmation of the neuron theory and of the idea of chemical neurotransmission in the central nervous system. Indeed, in 1954, Palade and Palay were the first to describe by electron microscopy that synapses were composed of a presynaptic terminal filled with vesicles opposed to a postsynaptic site (Palade and Palay, 1954). Later Katz, Del Castillo and Miledi hypothesized that the vesicles were filled with neurotransmitter and that their release occurred through the process of exocytosis. (Reviewed by (Wingate and Kwint, 2006; Lopez-Munoz and Alamo, 2009).

Constant progress in experimental techniques has permitted a better understanding of synaptic transmission. The identification of different neurotransmitters, multiple types of receptors and cascades of second messengers involved in the transduction of the synaptic signal led to a complex picture of the transfer of information from one neuron to another.

1.2- Synapse function: Overview

In the central nervous system, neurons communicate mainly through chemical synapses. The primary characteristics of synapses is to transmit electrical signals coming from the axon of one neuron, in the form of action potential, into chemical signals that will be converted back into electrical pulses in the dendrite of another neuron.

The first detailed description of the chemical synapse ultrastructure was performed in 1954 by Palay and Palade using electron microscopy (Palade and Palay, 1954). They showed that synapses are composed of pre- and postsynaptic compartments separated by a small gap, the synaptic cleft. The pre-synaptic terminal, which is a specialised enlargement of the axon, contains small clear synaptic vesicles filled with specific neurotransmitters (Fig. 1.1). When an action potential arrives at the nerve terminal it causes Ca^{2+} influx through the opening of voltage-gated Ca^{2+} channels. In turn, this triggers the fusion of synaptic vesicles with the plasma membrane at the active zone and the release of neurotransmitter into the synaptic cleft. Neurotransmitters then bind to specific postsynaptic receptors which, for fast synaptic transmission, are ligand-gated ion channels anchored to the electron-dense protein matrix that is the postsynaptic density (PSD). The subsequent opening of these ligand-gated ion channels leads to a change in potential at the postsynaptic membrane, giving rise to postsynaptic potentials (PSPs). PSPs are transmitted along the dendritic tree towards the soma, where they affect the excitability of the somatic membrane. Integration of multiple PSPs generated simultaneously by different presynaptic neurons eventually drives the initiation of action potentials at the axonal initial segment.

In the central nervous system, synapses mediate fast neurotransmission, which is highly temporally and spatially regulated. The close proximity of the pre and postsynaptic terminal permits a rapid transfer of neurotransmitters to their specific postsynaptic receptors. Furthermore, the recapture of neurotransmitter from the synaptic cleft by neuronal and/or glial cells serves to terminate synaptic transmission and further restrict the diffusion of the signal (Masson et al., 1999).

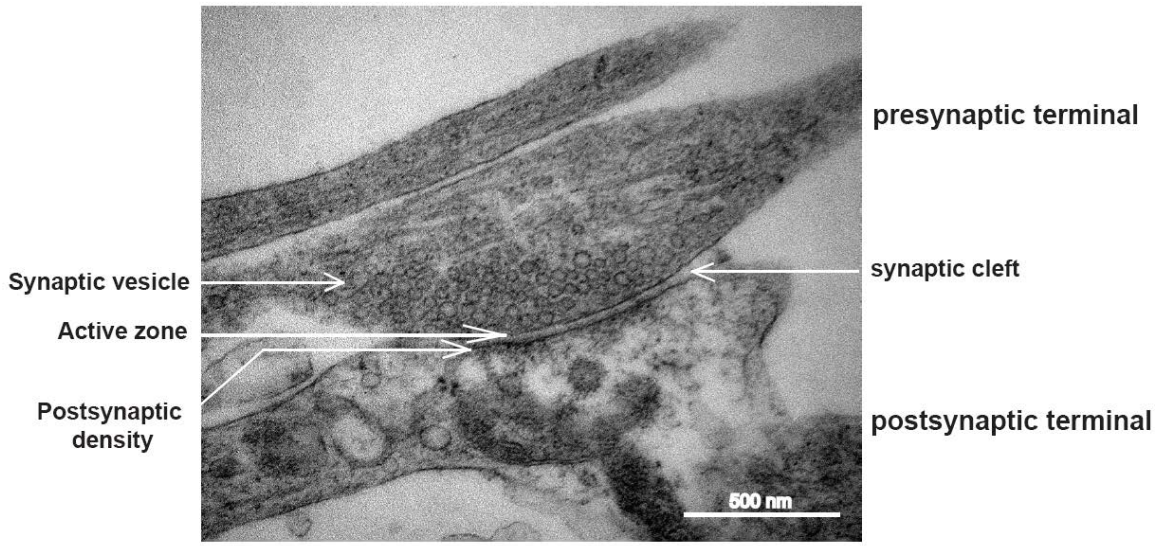


Figure 1.1: Ultrastructure organisation of the presynatic nerve terminal: Electron microscopy micrograph obtained from a 14 DIV hippocampal neuron in culture. The presynaptic terminal is packed with synaptic vesicles, some of which are in close proximity to the active zone. The active zone is well defined as an electron dense section of the presynaptic membrane that is directly apposed to another electron dense membrane structure: the postsynaptic density.

1.3- Release of neurotransmitter: Quantal theory

Fatt, Del Castillo and Katz pioneered our knowledge of synapse physiology (Fatt and Katz, 1952; Del Castillo and Katz, 1954). Using intracellular microelectrode recordings in the muscle cell of the frog, Fatt and Katz were the first to record the electrical response to acetylcholine (Ach) released at the neuromuscular junction, a phenomenon they called end-plate potential (EPP). In addition, they recorded small, spontaneous postsynaptic depolarizations that occurred stochastically in the absence of stimulation. They found that these spontaneous events shared common features with the EPP evoked by presynaptic stimulation, such as waveform shape and sensitivity to postsynaptic antagonists, and therefore named these events ‘miniature end-plate potentials’ (mEPP) or ‘minis’. Furthermore, this discovery coincided with the first ultrastructural identification of synaptic vesicles (Palade and Palay, 1954), which led Katz and colleagues to formulate the vesicular hypothesis for neurotransmitter release. This hypothesis stated that neurotransmitter was stored in vesicles and that the release of the content of each vesicle would elicit a unitary synaptic event that corresponded to the mEPP measured by electrophysiology (Fatt and Katz, 1952; Del Castillo and Katz,

1954). Del Castillo and Katz took these studies one step further by studying the relationship between these mEPPs and EPPs (Del Castillo and Katz, 1954). To do so, they artificially reduced the size of the EPP by incubating the neuromuscular junction in a solution containing low Ca^{2+} and higher than normal Mg^{2+} concentrations, a manipulation that effectively reduces the release probability at presynaptic boutons. Following stimulation, they mostly observed failures of neurotransmitter release (due to the low release probability), but when postsynaptic events were measured they were generally of small amplitudes, some of them comparable to mEPPs. The EPP amplitude frequency histogram obtained after multiple trials showed that the sizes of EPPs occurred in discrete quanta, which were multiples of the amplitude of the mEPP. In addition, this frequency distribution followed a Poisson law indicating that EPPs occurred stochastically and independently from one another. They concluded that the EPPs result from the synchronous release of multiple neurotransmitter quanta (Del Castillo and Katz, 1954; Augustine and Kasai, 2007). One assumption made in this model of quantal release, is that evoked and spontaneous neurotransmitter release use the same set of vesicles or that the vesicles are sufficiently similar and activate the same number of postsynaptic receptors in each case. Although the latter is highly likely, the former has recently been called into question and will be the focus of this thesis.

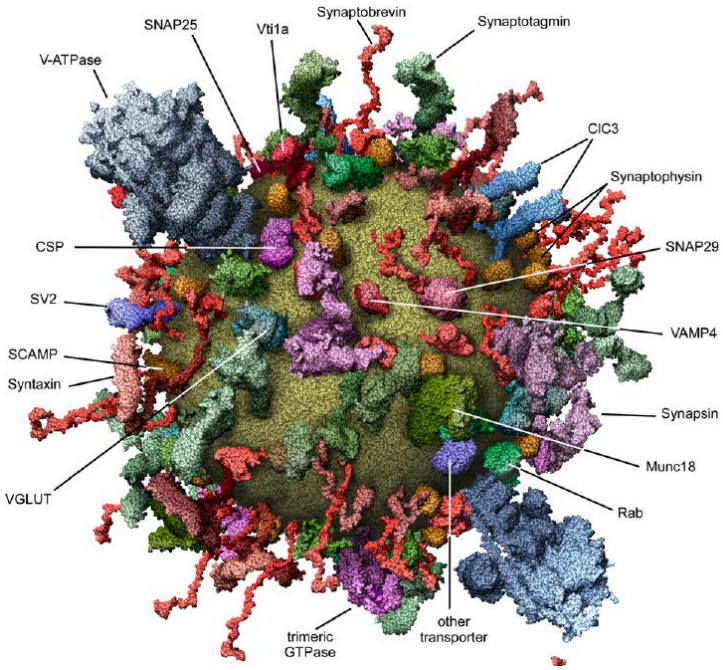
1.4- The presynaptic terminal: vesicles, pools and molecules

The pre-synaptic nerve terminal is a specialised enlargement of the axon where synaptic neurotransmission occurs. In this section I will describe the key elements that support neurotransmitter release, such as small clear synaptic vesicles and their organisation into distinct functional pools, the highly specialised active zone where exocytosis occurs and the specific set of proteins that are involved in calcium evoked exocytosis and endocytosis (Murthy and De Camilli, 2003).

1.4.1- Synaptic vesicles

Synaptic vesicles are the key elements in neurotransmission. The first electron microscopy images that allowed for the identification of small, uniform synaptic vesicles were done by Palade and Palay (Palade and Palay, 1954). Later, biochemical analysis allowed Whittaker to isolate the acetylcholine neurotransmitter from synaptic vesicles (Whittaker and Sheridan, 1965). Recently, a proteomic and lipidomic study has

permitted the first quantitative detailed characterisation of these synaptic vesicles at the molecular level (Takamori et al., 2006). Takamori *et al*, found that synaptic vesicles purified from rat brain have an average diameter of about 42 nm (similar to the diameter observed in electron micrographs), contain about 1500-2000 neurotransmitter molecules (Takamori et al., 2006). Figure 1.2, represents a molecular model of the major synaptic proteins present on synaptic vesicles. A large variability in copy numbers of different proteins was found at the single synaptic vesicle level. For instance, 70 copies of synaptobrevin (VAMP2), an important SNARE protein implicated in vesicle fusion with the plasma membrane, were found while only one copy of the proton pump, which serves to refill vesicles with neurotransmitter, was present. This large and diverse population of proteins participates in the complex regulation of synaptic vesicle function.



Shiego Takamori *et al.* 2006. Cell

Figure 1.2: Molecular model of a synaptic vesicle. Outside view of a vesicle with a realistic representation of all membrane proteins present.

1.4.2- Synaptic vesicle pools

The cortical nerve terminal contains about 200 synaptic vesicles that are thought to be organized into different pools according to their mobility in response to stimulation

(Sudhof, 2000; Rizzoli and Betz, 2005): the readily releasable pool (RRP), the reserve pool (RP) and the resting pool. The RRP is thought to correspond to the vesicles docked at the presynaptic active zone and primed for release (Schikorski and Stevens, 1997). These vesicles are immediately available for release in response to the arrival of an action potential (Rosenmund and Stevens, 1996). This pool contains a relatively small number of vesicles. For instance, the active zone of cultured hippocampal neurons synapses has about 8 to10 docked vesicles (Schikorski and Stevens, 2001). The RP is a pool of vesicles recruited in response to more sustained stimulation and serves to refill the RRP. Photoconversion of vesicles stained with the styryl dye FM1-43, together with electron microscopy, have established that the entire recycling pool represents only a subset (20 to 60%) of all the vesicles found at a presynaptic bouton in cultured hippocampal neurons (Harata et al., 2001). The RRP and the RP constitute what is known as the recycling pool, which includes all vesicles that recycle in response to neuronal activity. The remaining vesicles, those that cannot be mobilised in response to neuronal activity, constitute the resting pool (Fig. 1.3) (Harata et al., 2001; Sudhof, 2004). The function of this pool remains largely unknown (Sudhof, 2000).

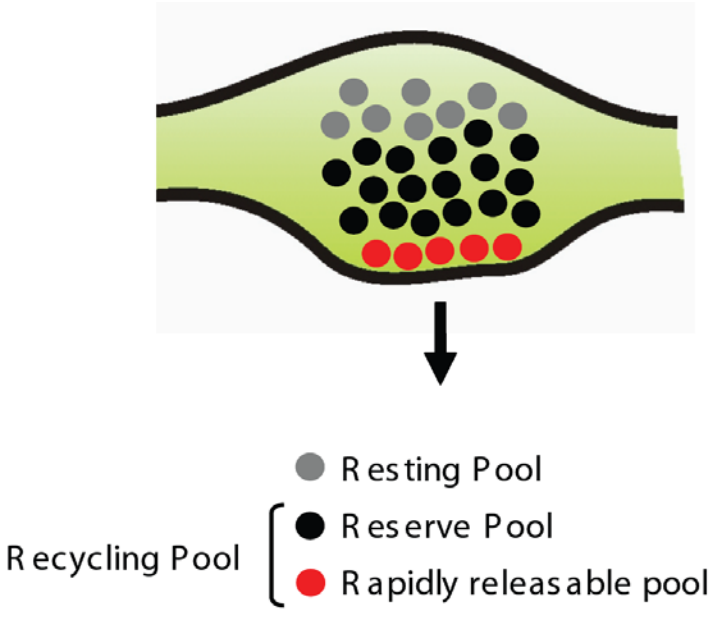
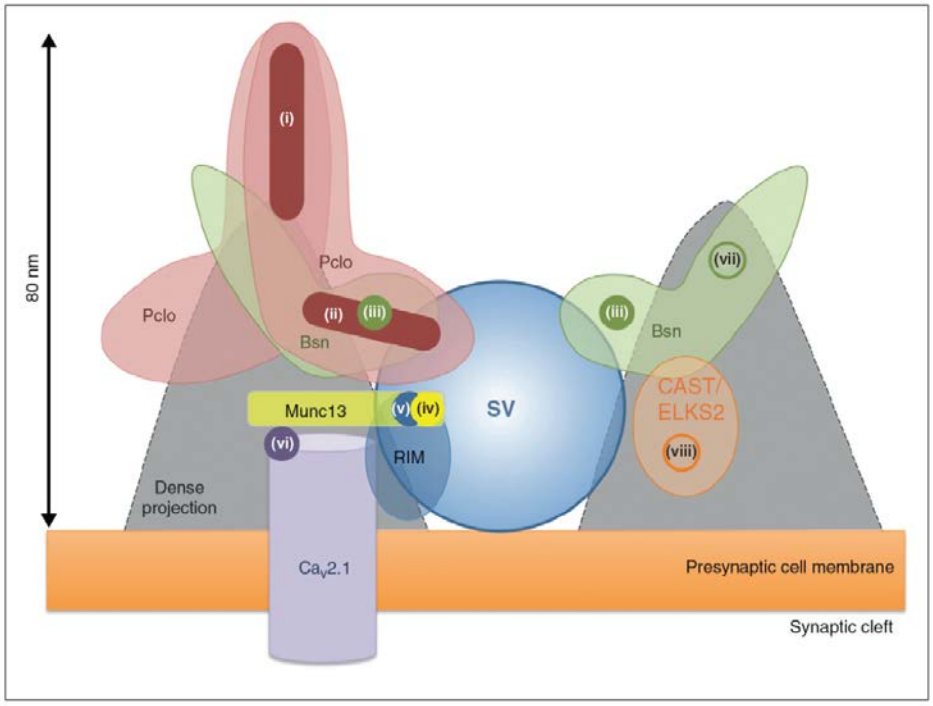


Figure 1.3: Synaptic vesicles pools: Schematic diagram represents a presynaptic terminal containing vesicles. These synaptic vesicles are organised into three functionally distinct pools: the readily releasable pool (RRP), the reserve pool (RP) and the resting pool. The RRP and the RP recycle in response to neuronal activity and thus form the recycling pool. The resting pool is made of vesicles that cannot be mobilised in response to neuronal activity.

1.4.3- Active zone

The active zone is a highly specialised site for neurotransmitter release at the presynaptic nerve terminal (Couteaux and Pecot-Dechavassine, 1970). It is part of the presynaptic membrane and is characterised by an electron dense structure, clearly visible by EM, which suggests it is composed of a rich protein matrix (Fig. 1.1). Indeed, the active zone membrane has a set of intracellular specialised proteins known as the cytomatrix of the active zone (CAZ) or presynaptic grid (Schoch and Gundelfinger, 2006; Gundelfinger and Fejtova, 2011). Among these, six main noticeable protein families have been characterised: Munc13s, Rab3-interacting molecules (RIMs), RIM-binding proteins (RIM-BPs), CAST/ELKS proteins, Piccolo and Bassoon, and the liprin-α family. These scaffolding proteins play a role in recruiting vesicles to the plasma membrane as well as modulating neurotransmitter release (for review see (Murthy and De Camilli, 2003; Schoch and Gundelfinger, 2006; Gundelfinger and Fejtova, 2011). Figure 1.4 illustrates the complexity of the interaction between the CAZ proteins. A small number of synaptic vesicles are clustered and tethered to the active zone and are thought to constitute the readily releasable pool (Couteaux and Pecot-Dechavassine, 1970; Heuser and Reese, 1973; Zenisek et al., 2000; Schikorski and Stevens, 2001). The active zone is precisely aligned with the postsynaptic density (PSD) so that after release, high concentrations of neurotransmitters reliably activate postsynaptic ionotropic receptors (see figure 1.1) (Zhai and Bellen, 2004). The active zone is therefore a critical part of the presynaptic terminal, acting as the main organizer of vesicle cycling.



Gundelfinger *et al.* 2011, Curr Opin Neurobiol.

Figure 1.4: Molecular and ultra-structural organization of the CAZ proteins at the active zone of mammalian small central synapses. The schematic diagram of the active zone was obtained following a reconstruction from immunogold localization of Bassoon (Bsn), Piccolo (Pclo), CAST/ELKS2, RIM, Munc13 and P/Q-type channel $Ca_v2.1$. The CAZ proteins participate in the recruitment of synaptic vesicles to the plasma membrane and in the modulation of neurotransmitter release.

1.5- The synaptic vesicle cycle

Presynaptic terminals contain about 200 neurotransmitter-filled small clear vesicles. In order to avoid depletion of the finite number of vesicles in response to sustained stimulations, they need to be recycled to allow for a constant supply of vesicles and provide the capacity to continuously release neurotransmitter when needed.

1.5.1- Overview of the synaptic vesicle cycle

At rest, there are a number of vesicles that are docked at the plasma membrane, primed and ready for release. When an action potential arrives at the presynaptic terminal it activates voltage-dependent Ca^{2+} channels resulting in an elevation of intracellular Ca^{2+}, which in turn causes neurotransmitter-filled vesicles to fuse partially or completely with the active zone plasma membrane and to release neurotransmitters into the synaptic cleft. Neurotransmitters bind to postsynaptic receptors resulting in the transfer of

information from one neuron to the next. Following exocytosis, the vesicular membrane and its associated proteins are retrieved by endocytosis. A proton pump (V type-ATPase) lowers the pH inside the vesicles and allows co-transporters to use this proton gradient to refill vesicles with neurotransmitter, ready for another round of release. Different ways of retrieving synaptic vesicles have been reported. In one form of membrane exo-endocytosis, synaptic vesicles fuse and fully collapse with the presynaptic membrane and are subsequently endocytosed at the periactive zone via clathrin-coated pits. Thereafter, they are either recycled directly (Li and Murthy, 2001) or through an endosomal compartment (Heuser and Reese, 1973). Alternatively, synaptic vesicles can fuse transiently with the plasma membrane and release their content through a fusion pore without full collapse of the vesicle. This mode of recycling is known as "kiss-and-run" (Ceccarelli et al., 1973). In this case, vesicles retain their molecular identity and do not mix with plasmatic or endosomal membrane compartments (see figure 1.5).

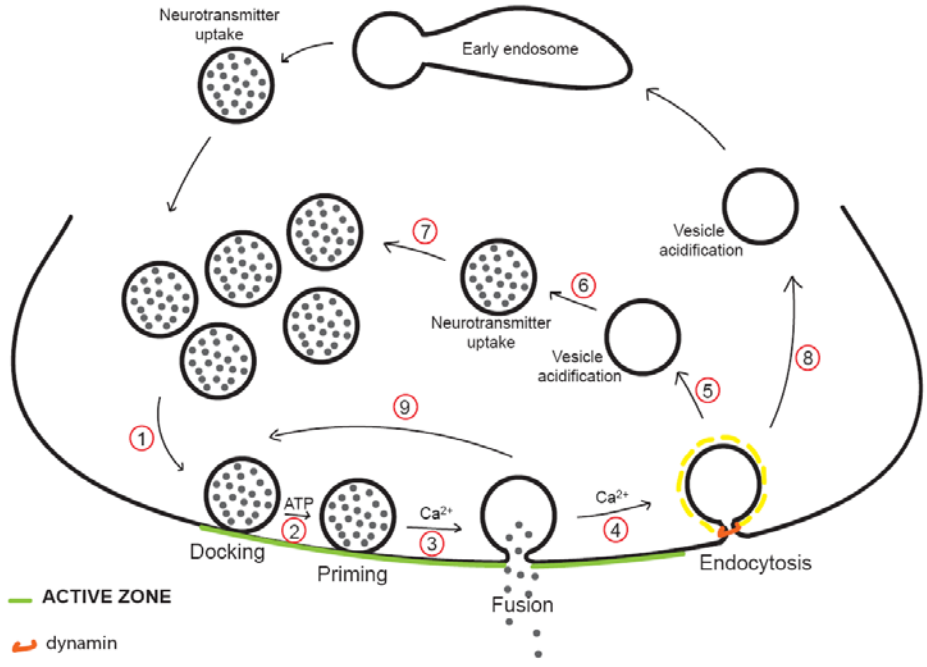


Figure 1.5: Synaptic vesicle cycling. At rest a number of vesicles are docked at the active zone①. Vesicles are then primed② and become competent for fusion. The arrival of an action potential leads to Ca^{2+} influx in the presynaptic terminal which in turn triggers vesicle fusion③. Vesicles are then retrieved outside the active zone via clathrin mediated endocytosis (CME)④. After reacidification⑤, newly formed vesicles are refilled with neurotransmitter⑥ and transported back to a pool of synaptic vesicles⑦ awaiting for a new round of exocytosis. Following CME, vesicles can alternatively recycle via endosomal compartments⑧. Vesicles can also fuse transiently and release their content through a transient pore; a pathway known as "kiss-and-run "⑨.

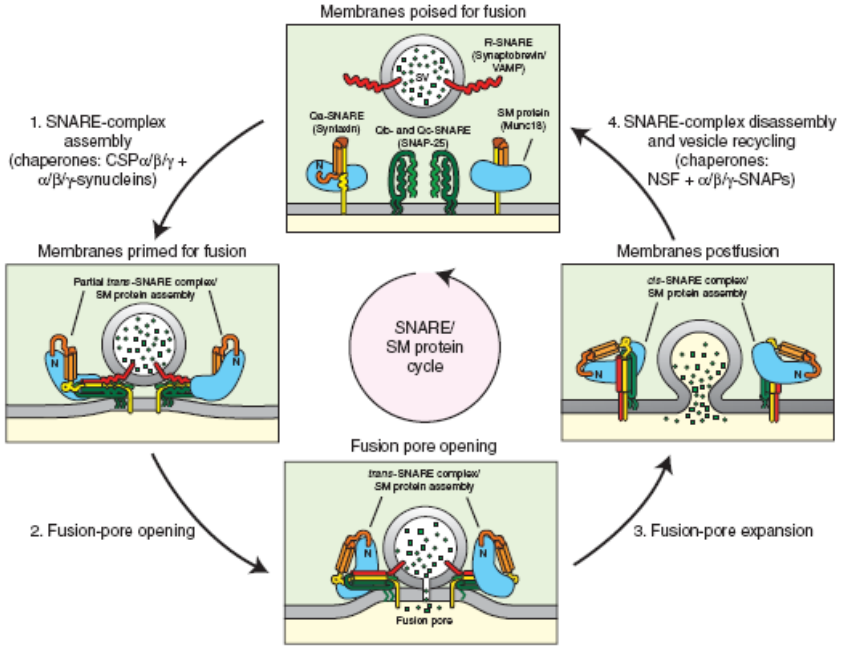
1.5.2- Exocytosis

Before synaptic vesicles fuse with the plasma membrane to release their content, they have to go through a process of maturation to become fusion-competent. This involves the recruitment of synaptic vesicles to the plasma membrane, their docking and priming. This implicates multiple interactions between synaptic vesicle and presynaptic plasma membrane lipids and proteins, as well as with CAZ proteins.

1.5.2.1- Formation of the SNARE complex

Docking, priming and fusion of synaptic vesicles require the formation of a SNARE (soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor) complex between the v-SNARE protein synaptobrevin 2 (VAMP2) and the two t-SNARE proteins syntaxin-1A and SNAP-25. SNARE-proteins possess a characteristic sequence, called the SNARE motif that assembles into a tight four-helix bundle to form the SNARE complex. This complex involves the interaction between one SNARE helix motif from VAMP2 and syntaxin, and two from SNAP-25 (Sutton et al., 1998; Antonin et al., 2002). It has recently been shown that one to three SNARE complexes may be sufficient to promote membrane fusion of one vesicle (Mohrmann et al., 2010; van den Bogaart et al., 2010; Sinha et al., 2011). The formation and the stabilization of this four-helical trans-SNARE complex is under the control of the cytosolic SM proteins (Sec1/Munc18-like proteins) which are critical for controlling the fusion of synaptic vesicles (Khvotchev et al., 2007; Gerber et al., 2008; Rathore et al., 2010). For instance, deletion of Munc18 in mice leads to a complete loss of vesicle fusion and neurotransmission (Verhage et al., 2000). However, the precise molecular interactions between SM proteins and the different components of the SNARE complex are still under investigation (Sudhof and Rizo, 2011).

Following fusion, trans-SNARE complexes are transformed into a cis-SNARE complex, which are then disassembled into monomers by the ATPase NSF and its co-factors SNAPs (soluble NSF attachment proteins). These reactive monomeric SNARE proteins are then recycled for another round of fusion (Fig. 1.6) (Jahn and Scheller, 2006; Sudhof and Rizo, 2011).



Sudhof and Rizo Cold Spring Harb Perspect Biol. 2011; 3:a005637

Figure 1.6: SNARE/SM protein cycle. SNARE proteins in the vesicle (VAMP2) and the plasma membrane (SNAP-25 and Syntaxin) interact to form the trans-SNARE complex during vesicle priming, with contribution of SM proteins, such as Munc18.

1.5.2.2- Preparing vesicle fusion: Role of CAZ proteins

CAZ proteins play a major role in orchestrating the release of synaptic vesicles. Munc 13 and RIM are the best characterized members of this group. Munc13 plays a central role in synaptic vesicle priming and Munc 13 knockout mice show a complete block of evoked and spontaneous neurotransmitter release (Augustin et al., 1999; Varoqueaux et al., 2002). This phenotype is not so surprising considering that Munc13 interacts with SNARE, SM proteins and RIM, all active zone proteins tightly involved in vesicle fusion events (Augustin et al., 1999). RIM, another important CAZ protein, is particularly critical for controlling neurotransmitter release. Originally identified as a Rab3a effector protein (Wang et al., 1997), it was also found to interact with scaffolding proteins partners such as α-liprin, RIM-BP and ELKS. RIM also binds to synaptotagmin, a major calcium-sensor protein on synaptic vesicles, and to voltage-gated Ca^{2+} channels (Sudhof and Rizo, 2011). In addition, RIM also plays a major role in recruiting Ca^{2+} channels to release sites and in defining the density of docked vesicles at the active zone. Deletion of RIM leads to severe defects in priming, a significant reduction of the size of the readily releasable pool of vesicles, a decrease in the number

of docked vesicles and a decrease in the amplitude of action potential-induced Ca^{2+} currents (Han et al., 2011; Kaeser et al., 2011). Overall, RIM has a central function in integrating active zone proteins and synaptic vesicles into a well organised web at defined release sites, thereby enabling fast neurotransmitter release (Fig. 1.7) (for review see (Schoch and Gundelfinger, 2006; Sudhof and Rizo, 2011).

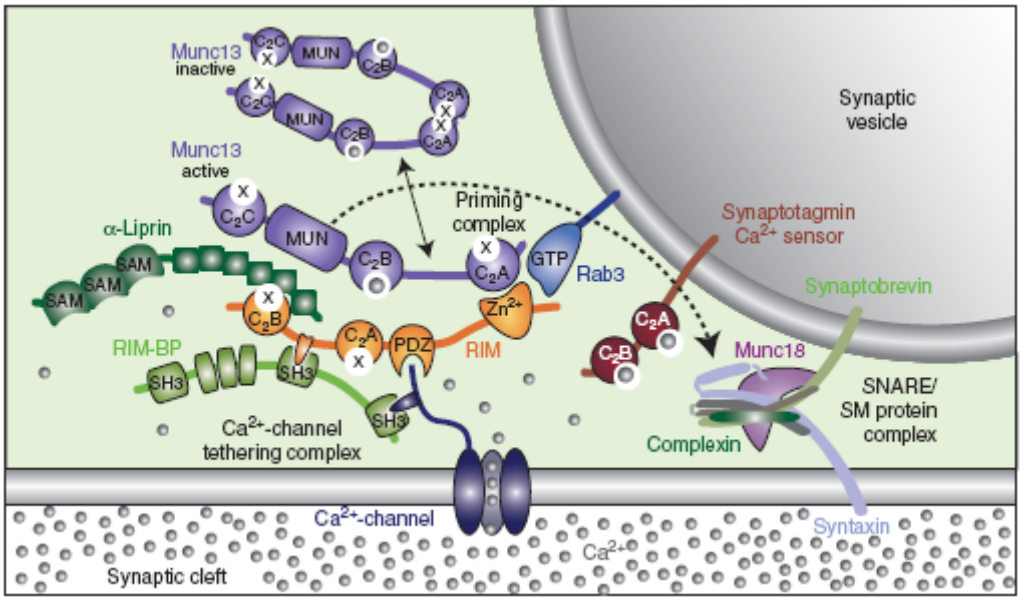


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Figure 1.7: Interaction of CAZ and synaptic vesicle proteins. Multiple scaffolding CAZ proteins, such as Munc 13 and RIM, interact to form an active zone complex that binds to synaptic vesicles and ensure their colocalization with other presynaptic components essential for neurotransmission, such as calcium channels.

1.5.2.3- Triggering vesicle fusion

Upon the arrival of an action potential, Ca^{2+} enters the presynaptic terminal through voltage-gated Ca^{2+} channels, and binds to a Ca^{2+} sensor that triggers vesicle fusion and neurotransmitter release. It is now well accepted that the vesicular protein synaptotagmin1 (Syt1) is the main Ca^{2+} sensor that mediates synchronous neurotransmitter release. Accordingly, hippocampal neurons from Syt1 knockout mice showed a dramatic decrease in fast synchronous neurotransmitter release (Geppert et al., 1994). Syt1 is one of 15 synaptotagmins that are encoded by different genes (Chapman, 2008). They contain a single N-terminal transmembrane domain and two C-terminal C2 domains, C2A and C2B, which are involved in a Ca^{2+}-dependent interaction with the

membrane and with the SNARE complex. Prior to Ca^{2+} entrance, the binding of the cytosolic protein complexin brings the SNARE complex into an activated state ("superprimed") but also prevents its complete assembly and subsequent membrane fusion. After Ca^{2+} influx, a Ca^{2+}-triggered change in conformation allows Syt1 to bind to the phospholipids of the plasma membrane and increases its affinity for the SNARE complex. This results in the displacement of complexin from the SNARE complex and triggers vesicle fusion (Fig. 1.7) (Giraudo et al., 2009; Maximov et al., 2009). In agreement with this mechanism, knockout mice of both complexin1 and 2 show a drastic decrease in evoked neurotransmitter release in hippocampal neurons (Reim et al., 2001).

1.5.3- Endocytosis

Following exocytosis, the vesicular membrane and its associated proteins are retrieved by compensatory endocytosis. Three modes of membrane and protein retrieval have been proposed to operate at the synapse: (1) a slow clathrin-mediated pathway (Heuser and Reese, 1973), (2) a fast track mode referred to as "kiss-and run" (Ceccarelli et al., 1973) and (3) activity-dependent bulk endocytosis (Richards et al., 2000).

1.5.3.1- Clathrin mediated endocytosis (CME)

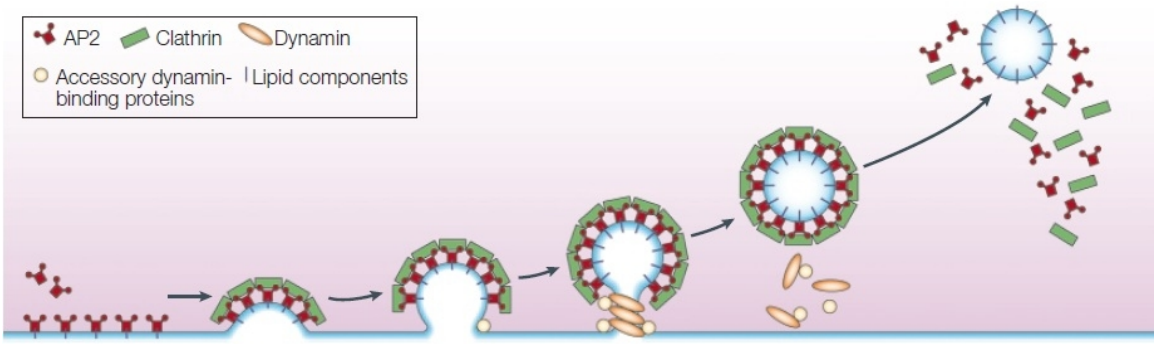
To date, CME is the major and most characterised mode of endocytosis. It was first described by Heuser and Reese when studying the ultrastructure of the frog neuromuscular junction (Heuser and Reese, 1973). They found that after a strong stimulation, presynaptic boutons showed a decrease in the number of synaptic vesicles, an increase in the number of coated vesicles and an increase in endosomal-like membrane compartments known as cisternae. After stimulation and a period of rest, the number of small vesicles increased and the membranous cisternae slowly disappeared. From these observations, Heuser *et al.* suggested that during exocytosis, vesicles collapse fully into the plasma membrane and are retrieved via coated pits. Later, a biochemical analysis identified the clathrin protein as being one of the major components of the coat described by Heuser and Reese (Pearse, 1976). Since then, these coated vesicles have been referred to as clathrin-coated vesicles.

In the current model of CME, the retrieval of exocytosed synaptic vesicle proteins starts with the recruitment of clathrin adaptor proteins (APs) that interact with the plasma membrane phospholipids and membrane cargo proteins that contains sorting motifs. Among clathrin-APs, the best characterized is AP-2. Biochemical studies have shown

that AP-2 interacts with synaptotagmin1 (Zhang et al., 1994). This and the severe impairment of vesicle endocytosis observed after inactivation of Syt1 demonstrate that this sensor is necessary for compensatory synaptic vesicle endocytosis (Poskanzer et al., 2003). The recruitment of AP-2 to the plasma membrane triggers the assembly of a clathrin cage in a process that induces the formation of a membrane invagination called coated pit. The formation of the coated pit requires a number of additional proteins such as AP-180 and endophilin. The coated pit then separates from the plasma membrane, thus generating free clathrin-coated vesicles. Fission of clathrin-coated vesicles from the plasma membrane is mediated by the GTPase dynamin. This protein clusters at the neck of the budding vesicle to form a multimeric helical structure that wraps around the invaginated membrane. Upon GTP hydrolysis, a conformational change occurs that results the vesicle detaching from the membrane through a mechanism that remains controversial and may involve either stretching or constriction of the helical structure (Ferguson and De Camilli, 2012). Once internalized and before being refilled by neurotransmitters, clathrin-coated vesicles have to be uncoated in an ATP-dependent process that requires auxilin and synaptojanin1. (Fig. 1.8) (For review see (Slepnev and De Camilli, 2000; Murthy and De Camilli, 2003; Dittman and Ryan, 2009)).

Although the process of exocytosis occurs within the restricted domain that is the active zone, CME was shown to occur at a different site, in the periactive zone (Gundelfinger et al., 2003). This region, that surrounds the active zone, is characterised by the presence of endocytic proteins such as intersectin, EPS15, dynamin and endophilin. This mechanism implicates that, following exocytosis and the collapse of the vesicle in the plasma membrane, synaptic vesicle proteins diffuse from the active zone to the periactive zone where they are recaptured (Roos and Kelly, 1999). This was confirmed in experiments using a combination of GFP and pHluorin-tagged VAMP2, showing that after vesicle fusion the synaptic vesicle protein VAMP2 migrated along the plasma membrane, away from the centre of the bouton, towards the periphery. Retrieval of VAMP2 through endocytosis occurred at these distant sites (Li and Murthy, 2001). Further evidence of this behaviour was observed when tracking the movement of clathrin together with other synaptic vesicle proteins during stimulation. Although initially restricted to the central domain of the presynaptic bouton, clathrin moved to the peri-synaptic area during a stimulus, as did other vesicle proteins (VAMP2 and synaptophysin) (Granseth et al., 2006). The emerging consensus in the field is that CME occurs away from the active zone and may represent a way to uncouple the process of exocytosis and endocytosis at the synapse.

A number of factors have been implicated in controlling the rate of endocytosis. CME has been shown to greatly depend on intracellular Ca^{2+} levels in hippocampal presynaptic terminals (Sankaranarayanan and Ryan, 2001). However, the time constant for endocytosis of individual vesicles was found to be insensitive to Ca^{2+}. Instead, Ca^{2+} was shown to increase the number of vesicle retrieved, providing a means for the synapse to match its endocytic capacity to its activity levels (Balaji et al., 2008). Another important factor modulating endocytosis is temperature, which unlike Ca^{2+} does affect the time constant for endocytosis. Indeed, work in cultured hippocampal neurons showed that CME occurs with a time constant of 15s at room temperature (25°C) and between 6 and 10s at physiological temperature (35°C) (Granseth et al., 2006; Balaji and Ryan, 2007). Altogether, this data suggests that CME should be sufficient to maintain synaptic transmission during sustained neuronal activity.



Gundelfinger *et al*. Nat Rev Mol Cell Biol.2003

Figure 1.8: Clathrin-mediated endocytosis (CME). The recruitment of clathrin adaptor proteins AP-2 to the plasma membrane triggers the assembly of a clathrin cage in a process that induces the formation of a membrane invagination called coated pit. Following the formation of the coated pit, dynamin is recruited to form a ring around the neck of the pit which induces the fission of the coated pit. Finally, clathrin-coated vesicles are uncoated and this requires proteins such as auxilin and synaptojanin. The uncoated vesicle is then refilled with neurotransmitter and ready for a new round of exocytosis.

1.5.3.2- Kiss-and-run

Ceccarelli *et al.* (Ceccarelli et al., 1973) proposed a different mode of endocytosis that did not rely on clathrin coats. Following low frequency stimulation of the frog neuromuscular junction, electron micrographs showed no endocytic intermediates nor any vesicle depletion at the terminal, suggesting that neurotransmitter could be released

without full collapse of vesicles. In this mode of endocytosis, later called kiss-and-run, vesicles release neurotransmitter through a transient fusion pore and are retrieved intact, without loss of their identity, ready for a new round of exocytosis. Kiss-and-run has been described to occur rapidly, in about 1 second, and mainly in response to low frequency stimulation (Pyle et al., 2000; Harata et al., 2006; Zhang et al., 2009). Perhaps the most direct evidence for kiss-and-run was shown in chromaffin cells where both capacitance (measuring the membrane surface area) and amperometry (quantifying neurotransmitter release) recordings of single large-dense core vesicles allowed detailed descriptions of membrane fusion and neurotransmitter release events. In these cells, large-dense-core vesicles were found to connect transiently with the plasma membrane via a fusion pore from which catecholamine release was detected (Albillos et al., 1997). Kiss-and-run was also found to occur at the calyx of Held using capacitance recordings where it appeared as a "brief capacitance flicker" that lasted for up to 2 seconds (He et al., 2006). Although capacitance recordings have not been possible in small synapses so far, optical approaches have been used to measure vesicle cycling with fluorescent styryl dyes (FM dyes) and pHluorin based probes (see chapter III on how they work) at small hippocampal synapses. With these methods, kiss and run events were described as transient incomplete release events (Pyle et al., 2000; Harata et al., 2006; Zhang et al., 2009). However, other studies did not confirm these observations (Granseth et al., 2006; Balaji and Ryan, 2007; Chen et al., 2008; Heerssen et al., 2008; Zhu et al., 2009). To date, the existence of kiss and run remain controversial and no clear molecular mechanism have been described.

1.5.3.3- Activity-dependent bulk endocytosis (ADBE)

A fast mode of endocytosis, known as bulk endocytosis, has been proposed to take place at the synapse during intense activity.

ADBE was first described by Miller and Heuser at the frog neuromuscular junction (Miller and Heuser, 1984). Since then, ADBE has been shown at the Calyx of Held (de Lange et al., 2003), in retinal bipolar neurones (Holt et al., 2003) and in primary hippocampal, cortical and cerebella neuron cultures (Evans and Cousin, 2007; Hayashi et al., 2008). During bulk endocytosis, a large area of the plasma membrane is internalized to form endosome-like structures. Coated vesicles are then retrieved from these structures and subsequently join the reserve pool of synaptic vesicles (Fig. 1.9) (Richards et al., 2000; Cheung et al., 2010). This mode of endocytosis has been found to occur only during periods of increased neuronal activity and is thought to ensure the fast

recapture of vesicles to help maintain intact the structure of the presynaptic terminal (Holt et al., 2003; Clayton et al., 2008). ADBE is triggered rapidly after the initiation of high frequency stimulation and ends as soon as the high frequency synaptic activity terminates. This is in contrast to CME that would persist for minutes after a strong stimulus (Clayton et al., 2008). The molecular mechanisms involved in ADBE still remain unknown, although Calcineurin has been suggested to be the Ca^{2+}sensor that triggers ADBE (Deak et al., 2004). Upon activation by Ca^{2+}, calcineurin induces the activation of dynamin which in turn drives the formation of the endosomal-like structures and the budding of synaptic vesicles from these endosomes (Evans and Cousin, 2007; Clayton and Cousin, 2009).

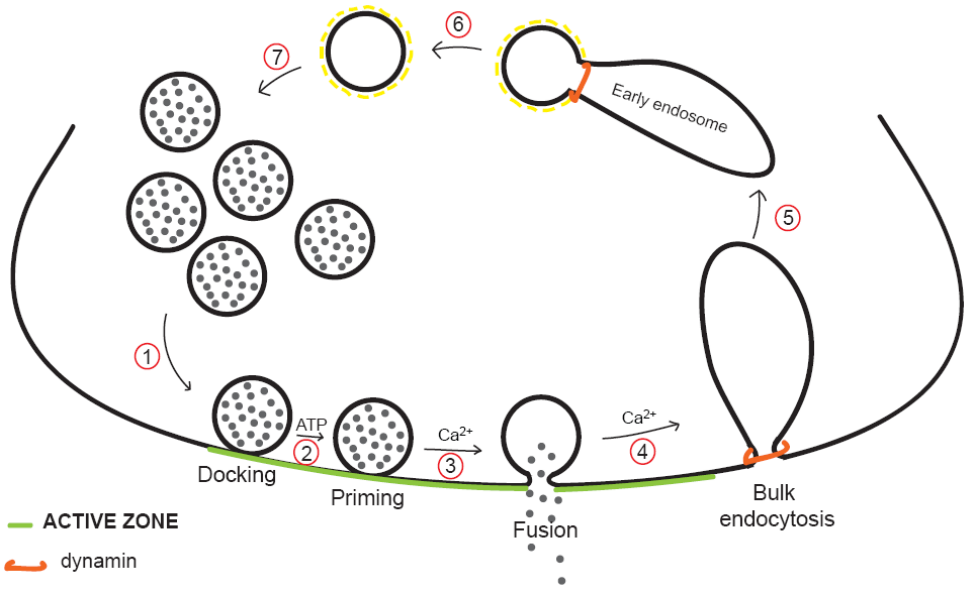


Figure 1.9: Activity-dependent bulk endocytosis (ADBE). At rest a number of vesicles are docked at the active zone①, vesicles are then primed② and become competent for fusion. Strong stimulations lead to Ca^{2+} influx inside the presynaptic terminal which in turn triggers vesicle fusion③. Vesicles are then retrieved outside the active zone via bulk endocytosis which consists of the invagination of large areas of presynaptic membrane.④. This result in the formation of endosomes ⑤ from which coated-vesicles bud ⑥ .Newly formed vesicles are uncoated, reacidified, filled with neurotransmitter and transported back to the cluster of synaptic vesicles⑦ for a new round of exocytosis.

1.6- Spontaneous versus activity-dependent neurotransmitter release

Following an action potential, neurotransmitter is released via the synchronous fusion of vesicles with the plasma membrane. This modality is also referred to as activity-dependent or evoked release. However, neurotransmitter release can also occur in the absence of action potentials, a process known as spontaneous release. All synapses studied to date undergo evoked (action potential-dependent) and spontaneous (action potential-independent) release (Del Castillo and Katz, 1954). While evoked release is highly spatially and temporally regulated (Sabatini and Regehr, 1999), spontaneous release happens stochastically with a very low frequency (Murthy and Stevens, 1999). The general view on evoked vesicle fusion has generally been that calcium influx increases the probability of fusion of vesicles that can also be released spontaneously. At rest, in the absence of any activity, the machinery that is responsible for the fusion of neurotransmitter-filled vesicles at a synapse has a very low release probability, but which is not zero. As a result, spontaneous fusion events occur stochastically, but at a very low rate, estimated to be around 1–2 vesicle per min (Geppert et al., 1994; Murthy and Stevens, 1999). The consensus view is that membrane depolarization (by action potentials or otherwise) increases the probability of vesicle fusion, allowing the synapse to switch from a low rate of spontaneous release to a rapid synchronized form of release. This also means that both spontaneous and evoked release relies on one pool of vesicles using the same release machinery. However, there is now accumulating evidence suggesting that evoked and spontaneous release arise from independent pools of vesicles with different release mechanisms.

1.6.1- Evidence for a distinct molecular machinery responsible for evoked and spontaneous neurotransmitter release

As described above, the SNARE complex that mediates evoked neurotransmitter release has been well characterized and is composed of the SNARE proteins VAMP2, syntaxin-1 and SNAP-25. Studies that have systematically modified any of these proteins, either by means of knock outs, knock downs or targeted mutations have resulted in varying effects on evoked and spontaneous release. For instance, a loss of VAMP2 or SNAP-25 resulted in an important reduction of evoked neurotransmitter release while spontaneous release persisted, albeit at a lower frequency (Schoch et al., 2001; Washbourne et al., 2002). Similarly, in Drosophila lacking syntaxin, evoked release was drastically diminished while spontaneous release still occurred (Schulze et al., 1995). These

findings suggest that different fusion complexes might be implicated in these two forms of release. An alternative possibility is that the same SNARE proteins are required for spontaneous and evoked release but that they differ in their mode of interaction. Indeed, the insertion of 12 residues between the SNARE motif and the transmembrane region of a VAMP2 construct resulted in the rescue of spontaneous release when expressed in synaptobrevin-2 deficient neurons, while evoked release was still impaired (Deak et al., 2006). Altogether, these observations suggest that the fusion mechanisms of spontaneous and evoked release may differ, but further investigation is necessary to define their molecular identity.

1.6.2- Calcium-dependence of spontaneous neurotransmitter release

As shown earlier, evoked synaptic vesicle release requires Ca^{2+} and depends on the presence of a Ca^{2+} sensor. Similarly, spontaneous release was shown to be highly Ca^{2+}-dependent. Several studies have shown that both extracellular Ca^{2+} (Llano et al., 2000; Xu et al., 2009) and internal Ca^{2+} stores (Llano et al., 2000; Emptage et al., 2001) influence spontaneous activity. This prompts the question of whether evoked and spontaneous vesicle fusion depend on a same Ca^{2+} sensor.

It is now well established that evoked neurotransmitter release requires Syt1 as a Ca^{2+} sensor for vesicle fusion (Geppert et al., 1994). However, in the Drosophila neuromuscular junction (DiAntonio et al., 1993; Littleton et al., 1994) and at mammalian central synapses (Maximov and Sudhof, 2005), a lack of Syt1 resulted in an increase in spontaneous release, as well as the expected loss of evoked release. This result, showing opposite effects on the two forms of release, suggests that Syt1 may operate as a fusion clamp rather than as a Ca^{2+} sensor for spontaneous release. In other words, Syt-1 may actually hold back docked vesicles from fusing with the membrane, until calcium influx occurs. In another set of experiments, Jun Xu *et al.* used point mutations in the Ca^{2+} binding site of Syt1 to show that this sensor controls both evoked and spontaneous release (Xu et al., 2009). Furthermore, they demonstrated that the paradoxical increase in spontaneous release in the Syt1 knockout was due to a second high-affinity Ca^{2+} sensor which is clamped by Syt1 in physiological conditions, although they did not uncover the identity of this sensor (Xu et al., 2009). In a more recent study, Groffen *et al.* identified a possible high-affinity calcium sensor, the double C2 domain 2b protein (Doc2b), which competes with Syt1 for binding to the SNARE complex. In contrast to Xu *et al.*, they proposed that this Ca^{2+} sensor and not Syt1 is driving spontaneous release (Groffen et al., 2010). However, in an even more recent

study, Pang *et al.* showed that removing the Ca^{2+} binding sites from Doc2b did not alter its ability to modulate spontaneous release (Pang et al., 2011). The same group also identified another Ca^{2+}-independent regulator of spontaneous release, synaptotagmin 12 (Syt12), which has been described as low affinity calcium sensor. Over-expression of this synaptotagmin isoform in neuronal cultures increased spontaneous neurotransmitter release without affecting evoked release (Maximov et al., 2007).

From the present data, it appears that both Ca^{2+}-dependent and Ca^{2+}-independent regulatory mechanisms exist for spontaneous release. Further investigations are needed to get a better understanding of the detailed molecular mechanisms driving and modulating spontaneous release.

1.7- Vesicle cycling during development

1.7.1- Synaptogenesis

The formation of most synaptic connections occurs during development, when neurons extend axons and establish connections with their postsynaptic targets. The acquisition of polarity, which implicates axonal and dendritic specification, occurs through five successive stages. This sequence of events was described in great detail by Dotti *et al.* (1988), in primary cultures of developing hippocampal neurons (Fig. 1.10a) (Dotti et al., 1988). Shortly after attaching to the substrate, motile lamellipodia form around the cell periphery (stage 1). Within the first 24 hours, lamellipodia then condense to form short neurites, all similar to each other, known as minor processes, which give rise to a stage characterized by the multipolar appearance of the neuron (stage 2). Then, one of these neurites begins to grow at a faster rate than the others and differentiates into an axon (stage 3). At this stage of development, the neuron has become 'polarized'. Two to three days after axonal outgrowth, the other minor processes differentiate and acquire the typical structural and molecular characteristics of dendrites (stage 4). Stage 5 corresponds to the cell-cell interactions which influence the subsequent maturation of both the axon and the dendritic tree with the formation of axonal terminals, dendritic spines and the appearance of functional synapses (see also for review: (Ledesma and Dotti, 2003)).

The process of synapse formation during axonal development has been studied in more detail in *in vitro* systems such as hippocampal cultures, by using presynaptic functional markers. Burrone *et al.* characterized the time course of synapse formation in cultured

hippocampal neurons with FM4-64 (Burrone et al., 2002), a fluorescent dye tagging synaptic vesicle release (Betz and Bewick, 1992). By 4 DIV, functional presynaptic terminals could be detected with a significant increase in their number between 6 and 10 DIV. After 10 DIV, the number of synapses increased moderately until reaching a plateau by 14 DIV (Fig. 1.10b) (Renger et al., 2001). Together, these findings show that hippocampal neurons grown in vitro develop by following a stereotyped sequence of events. This 2-dimensional system is therefore an especially amenable system to study synapse formation. During the first week of this in vitro preparation, very few synapses are made (Renger et al., 2001; Burrone et al., 2002). Instead, growth cones are actively searching for a postsynaptic partner, offering an ideal window of time to study vesicle dynamics in axons devoid of a postsynaptic partner. Most synapses form as a burst of synaptogenesis between day 6 and day 11 in vitro. This dynamic period offers another temporal window to understand how growth cones stabilize and mature to form synaptic connections with other neurons. Finally, after 2 weeks in vitro, synapses have stabilized and matured, allowing the study of vesicle cycling in functional networks (Burrone et al., 2002).

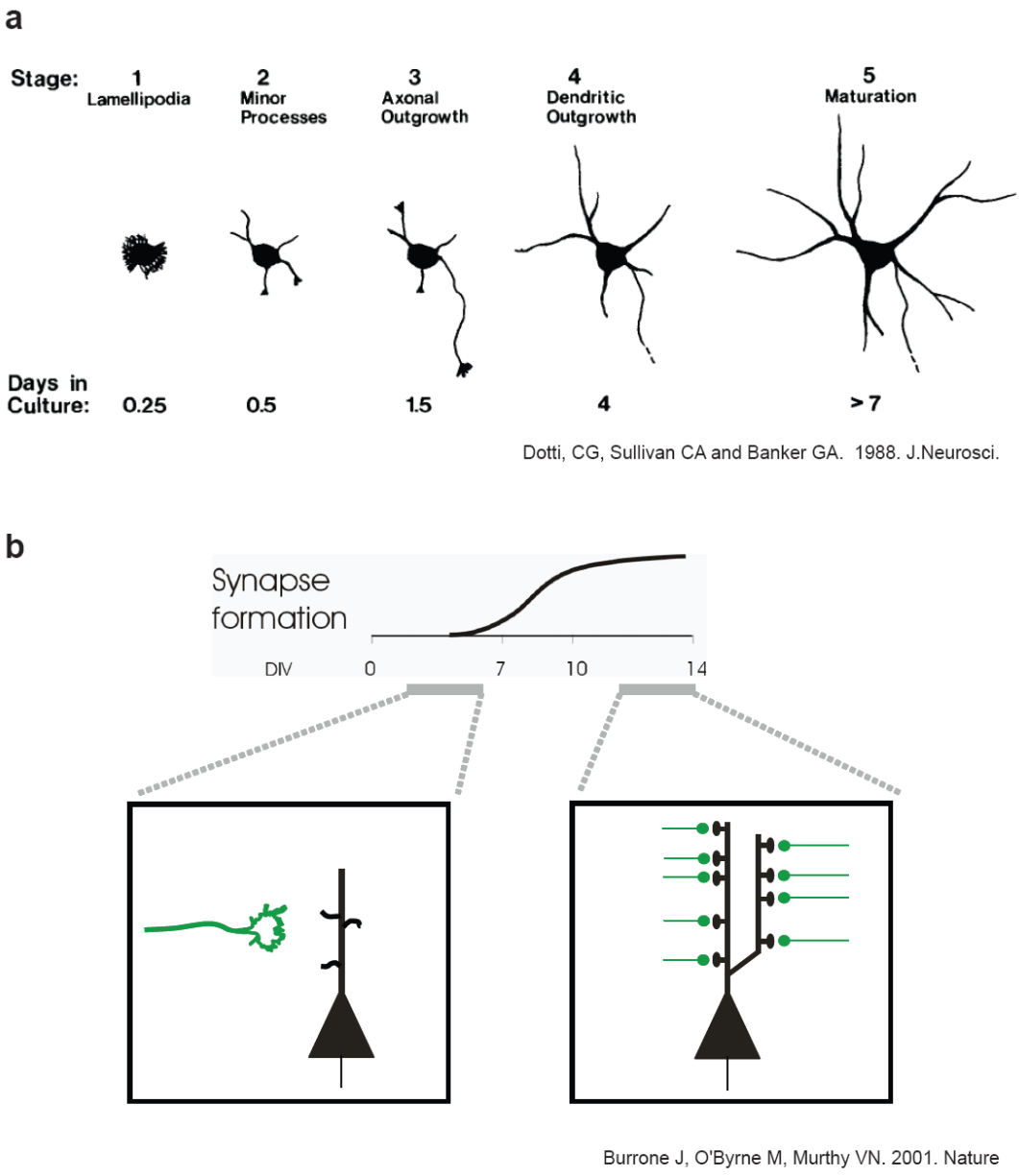


Figure 1.10: Neuron differentiation and synaptogenesis: (**a**) Stages of development of hippocampal neurons in culture (refer to 1.6.1 for discussion). (**b**) Time course of synapse formation of hippocampal neurons in culture. Before synapse formation, the growth cone navigates to find its postsynaptic partner. Synapses starts to form between 4 and 7 DIV and are fully mature by 14 DIV. The number of functional synapses was measured using the vesicle dye FM4-64.

1.7.2- Neurotransmitter release in immature neurons

Functional synaptic transmission has been detected within minutes to an hour after axodendritic contact in hippocampal neuronal cultures (Hume et al., 1983; Ahmari et al., 2000; Friedman et al., 2000; Lohmann and Bonhoeffer, 2008). The rapidity of the onset of regulated neurotransmitter secretion after synapse formation implies that neurons may have already acquired the appropriate machinery for neurotransmitter release before contacting the target cell (Young and Poo, 1983). Indeed, a number of studies using various preparations have shown that both spontaneous and evoked neurotransmitter release occurred in growing axons (Matteoli et al., 1992; Kraszewski et al., 1995; Dai and Peng, 1996; Nakata et al., 1998; Zakharenko et al., 1999; Sabo et al., 2006) and specifically at the growth cone (Hume et al., 1983; Young and Poo, 1983; Diefenbach et al., 1999), prior to synapse formation. Vesicles implicated in neurotransmitter release during neuronal development are different from the ones mediating axonal outgrowth. Indeed, it has been shown that mutation in *Sec5* (a protein of the exocyst complex) in drosophila neurons impaired the addition of newly synthesized proteins to the plasma membrane and neurons failed to extend axons, whereas synaptic vesicle fusion was not affected in mature synapses (Murthy et al., 2003). Furthermore, knockout mice lacking either Munc18 (Verhage et al., 2000) or Munc13 (Varoqueaux et al., 2002), two proteins implicated in synaptic vesicle fusion, resulted in a total loss of neurotransmitter release while synaptogenesis happened normally. Together, this data demonstrates that membrane expansion and neurotransmitter release happens through two distinct vesicular pathways. Indeed, Plasmalemmal Precursor Vesicles (PPVs) have recently been identified as the vesicles responsible for membrane expansion. They were described by electron microscopy as being clusters of large (about 150 nm diameter) pleiomorphic vesicles that were mainly seen in growing axons and dentrites, and in regenerating axons (Pfenninger, 2009). So far, the PPVs have not been characterized molecularly as no specific markers have been found to identify them. Therefore, their identification is mainly based on their morphology (Pfenninger, 2009).

1.7.3- Vesicle cycling in an immature neuron

Fletcher *et al.* (1991) were the first group to study the distribution of presynaptic vesicle proteins using immunofluorescent labeling during the development of hippocampal neurons in culture (Fletcher et al., 1991). They showed that at early stages of development, as soon as one neurite differentiates into an axon (at stage 3), both

synapsin 1 and synaptophysin were preferentially localized to the distal axon and its growth cone, appearing as punctate structures of various sizes. On the other hand, in more mature cultures, these synaptic vesicle proteins became clustered at the presynaptic terminal suggesting that their recruitment to a new synaptic site is initiated by the axo-dendritic contact (Fletcher et al., 1991). In fact, time lapse imaging of hippocampal neurons expressing VAMP2-GFP has revealed the presence of highly motile small heterogeneous puncta that moved in a retrograde and anterograde direction along the developing axon (Kraszewski et al., 1995; Nakata et al., 1998; Ahmari et al., 2000). These motile puncta were called 'transport packets' and were recruited upon contact with a postsynaptic partner (Ahmari et al., 2000). Ultrastructural analysis of these transport packets revealed the presence of 'dense-core vesicles', 'pleiomorphic small vesicles' and 'tubulovesicular structures', but structures resembling the small clear synaptic vesicles were only rarely observed (Nakata et al., 1998; Ahmari et al., 2000). They were shown to carry components of the mature presynaptic terminal, such as voltage-dependent calcium channel subunits, endocytic proteins and synaptic vesicle proteins (VAMP2, synapsin 1 and SV2). From these findings, it was suggested that the transport packets might be precursors to synaptic vesicles (Kraszewski et al., 1995; Ahmari et al., 2000).

Another type of presynaptic precursor has been described in developing axons, known as 'piccolo transport vesicles' (PTVs) (Zhai et al., 2001; Shapira et al., 2003). They are motile large dense-core vesicles and were shown to carry proteins of the active zone, such as the cytomatrix of the active zone (CAZ) scaffolding proteins, bassoon and piccolo. They were also shown to carry proteins implicated in synaptic vesicle exocytosis, including, syntaxin, SNAP-25, Munc13 and Munc18 (Zhai et al., 2001; Shapira et al., 2003). This is an intriguing mechanism for forming an active zone, where all its components are delivered in one large vesicle that fuses with the plasma membrane at its required site.

It has been shown that different motor proteins are responsible for transporting synaptic vesicle precursors and PTVs (Jin and Garner, 2008). Perhaps more surprisingly, these synaptic vesicle precursors have been shown to fuse transiently with the plasma membrane as they move along the axon and in the growth cone of developing neurons (Matteoli et al., 1992; Kraszewski et al., 1995; Dai and Peng, 1996; Diefenbach et al., 1999; Zakharenko et al., 1999; Sabo and McAllister, 2003; Tojima et al., 2007). Using the steryl dye FM1-43, Diefenbach *et al.*, have demonstrated the presence of two distinct pools of vesicles in the growth cone: one that recycle constitutively and another

that is mobilized in response to activity (Diefenbach et al., 1999). Interestingly, it was found that although young neurons (Div 3) showed robust spontaneous cycling, this form of release was then gradually down-regulated (Kraszewski et al., 1995; Coco et al., 1998), and an activity-dependent form of cycling was recruited at the nascent synapse (Ahmari et al., 2000). Until now, no complete study has investigated the different pools of vesicles and their modes of release during neuronal development. Furthermore, it is unclear what the role of neurotransmitter release early in development is. It has been suggested that it could contribute to synapse formation (Chang and De Camilli, 2001; Kwon and Sabatini, 2011). However, the targets of this neurotransmission remain to be identified. Plausible candidates are either the developing axon itself, in an autocrine fashion, neighboring axons or developing dendrites in the vicinity. Although glutamate receptors have been described in both axons and dendrites in developing neurons, clear evidence that distinguished between these possibilities does not currently exist.

1.8- Aims of the thesis

Synaptic vesicle cycling is important for maintaining synapse function. The aim of this study is to explore the different modes of synaptic vesicle recycling and the role of the different pools of vesicles in synapse function. For this purpose, dissociated rat hippocampal neuronal cultures will be used. This model offers the possibility to combine the use of genetically encoded probes and high resolution imaging to study synaptic function. Firstly, a new genetically-encoded probe, biosyn, which reports presynaptic activity will be developed and characterized. Using biosyn in combination with existing optical tools, the different modes of release and their pool of origin at the mature presynaptic terminal will be investigated. Finally, I will extend the knowledge and the use of all the techniques applied in mature systems into early development in order to characterize the different modes of release during axon growth and synapse formation.

CHAPTER II

Materials and Methods

2.1- Materials

2.1.1- Standard solutions for molecular biology

- **1% Agarose gel:** 1g agarose in 100 ml 1XTAE
- **Ampicillin** (Sigma): Stock solution is at 50 mg/ml: 5 g ampicillin in 100 ml distilled water, kept at -20°C. The working concentration is at 50µg/ml (diluted 1/1000).
- **Kanamycin** (Sigma): 50 µg/ml
- **LB Agar** Luria Agar (Sigma)
- **LB Broth** Luria Broth Base (Sigma)
- **TAE**: 40 mM Tris-Acetate, 1 mM EDTA

2.1.2- Plasmids and Expression Vectors

Insert	Vector	Tag	Source
GFP	pCAβ	-	Gift from B. Eickholt, MRC Centre for Development Neurobiology, KCl
Synaptophysin-pHluorin		pHluorin	Gift from L. Lagnado, Medical Research Council Laboratory of Molecular Biology, Cambridge
SynaptopHluorin	pCl	pHluorin	Gift from G. Miesenbock, Department of Physiology, Anatomy and Genetics, University of Oxford
BirA	pCDNA3	-	Gift from O. Burrone, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy
TeNT-Lc:EGFP	pEGFP-N2 (Clontech)	GFP	Gift from MP. Meyer, MRC Centre for Development Neurobiology, KCl
TeNTLcmut:EGFP	pEGFP-N2 (Clontech)	GFP	Gift from MP. Meyer, MRC Centre for Development Neurobiology, KCl
TeNT-Lc:TdT	pEGFP-N2 (Clontech)	TdT	Gift from MP. Meyer, MRC Centre for Development Neurobiology, KCl

2.1.3- Cell culture Media and Reagents

● ***General reagents and standard solutions***

- *HBSS* (Invitrogen) Hank's medium w/o Ca^{2+} and Mg^{2+}
- *Sterile PBS* (Sigma) Phosphate buffered saline, pH 7.3

● ***Coverslips***

- 18 mm glass coverslip (Menzel Gläser, Germany) were rinsed with a solution containing 60% ethanol and 40% HCl for 10 min at RT on a rotating shaker. They were then washed extensively with distilled water and rinsed with ethanol for 10 min. There were then washed with distilled water three times and left to dry. Finally, there were sterilised for 1 h at 180°C in a dry oven.

- 35 mm plates were used for CHO cell transfection

● ***Glass coverslips coating solution***

- *Poly-D-lysine* (Sigma) was dissolved in PBS and kept as stocks of 1 mg/ml and used at 50 µg/ml for coating of coverslips.

- *Laminin* (Sigma) was kept as stocks of 1mg/ml and used at 20 µg/ml for coating of coverslips

● ***CHO cell medium***

. DMEM medium (Invitrogen)	
. Foetal Bovine Serum	10%
. Penicillin/Streptomycin (Sigma)	1%

● ***Hippocampal cell medium***

- *Attachment medium*

. Neurobasal medium (Invitrogen)	
. B27 (Invitrogen)	2%
. Foetal Calf Serum (FCS) (Invitrogen)	2%
. Glutamax (Invitrogen)	1%
. Penicillin/Streptomycin (Sigma)	1%

- *Maintenance medium*

. Neurobasal medium (Invitrogen)	
. B27 (Invitrogen)	2%

. Glutamax (Invitrogen) 1%

. Penicillin/Streptamincyn (Sigma) 1%

● ***Hippocampal neuron dissociation reagent***

- *Trypsin* (Worthington) 1mg/ml diluted in HBSS

● ***Transfection reagent for Hippocampal neurons***

- *Lipofectamine 2000* (Invitrogen)
- *Opti-MEM* (Invitrogen)

●***Transfection reagent for CHO cells***

- Lipofectamine (Invitrogen)

● **Fixation solution**

Paraformaldehyde (Sigma), 4% in PBS (pH 7.4) and 0.33 M sucrose

● **Mounting solution**

. Mowiol 4-88 (Calbiochem) 5g

. PBS 20ml

. Glycerine 10ml

Mowiol was stirred overnight in PBS (room temperature), then glycerol was added. Glycerol mix was stirred for 16 more hours. Undissolved mowiol was centrifuged and the supernatant was kept in the -20°C in small aliquots. Before use, DABCO (Sigma) was added (anti-fade).

2.1.4- Standard solution and reagents for hippocampal neurons treatments

2.1.4.1- Extracellular medium: HBS (Hepes Buffered Saline)

● ***HBS solution*** containing (in mM): 139 NaCl, 2.5 KCl, 10 Hepes, 10 D-glucose, 2 $CaCl_2$, 1.3 $MgCl_2$, pH 7.3, 290 mOsmol.

● ***Depolarizing solution*** consists of HBS containing (in mM): 78.5 NaCl, 60 KCl, 10 Hepes, 10 D-glucose, 2 $CaCl_2$, 1.3 $MgCl_2$, pH 7.3, 290 mOsmol. 0.001 mM TTX,

0.025 mM APV and 0.02 mM (CNQX) were added to medium before treating the neurons.

● ***Spontaneous solution*** consists of Ca^{2+}-free HBS. 0.001 mM TTX, 0.025 mM APV and 0.02 mM (CNQX) were added to medium before treating the neurons.

2.1.4.2- Drugs

Drugs& specific labels	Action	Stock solution	Final concentration	Suppplier
APV (2-amino-5-phosphonovaleric acid)	NMDA receptor antagonist	25mM stock solution: 25mg APV in 5.2 ml distilled water	25 µM	Sigma
CNQX (6-cyano-7-nitroquinoxaline-2,3-dione)	AMPA receptor antagonist	20mM stock solution: 25mg CNQX in 4.525 ml distilled water	20 µM	Sigma
TTX (tetrodotoxin)	Voltage gated sodium antagonist	1 mM stock solution: 1mg in 3.1ml distilled water	1 µM	Alomone labs
Bafilomycin A	Blocker of the vesicular proton pump	0.3mM stock solution: 10µg in 32.1µl DMSO	1 µM	Calbiochem
Cadmium	Blocker of voltage-gated calcium channels	100mM stock solution	100 µM	Aldrich

2.1.4.3- Others standard reagents

● ***Biotin solution***: Powder stored at + 4°C. 100 mM stock solutions were prepared in phosphate-buffer saline (PBS) solution is kept at +4°C. Working concentration is 100 µM.

● ***Streptavidin Alexa*** dyes are used at a dilution of 1/250. Streptavidin is commercially available (Invitrogen) conjugated with different fluorophores. Any could be used depending on your excitation and emission filters. The ones used in this study are: Streptavidin-AlexaFluor555 (strep555: red), Streptavidin-AlexaFluor647 (strep647: far red), Streptavidin-AlexaFluor594 (strep594: red), Streptavidin-AlexaFluor488

(strep488: green), unlabelled Streptavidin and fluoroNanogold-streptavidin-Alexa Fluor488 (from Nanoprobes).

- ***FM4-64 FX***: [*N*-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl) hexatrienyl)pyridinium dibromide] (Invitrogen): 10 mM stock solutions were prepared in H2O and kept -20°C. Working concentration is 10 µM.

2.2- Methods

2.2.1- Molecular Biology

2.2.1.1- DNA electrophoresis

A 1% gel agarose in TAE gel containing 0.5µg/ml ethidium bromide was used to analyze DNA products diluted in 10X 'Orange G' loading dye to a final 1X concentration and run at 120 V with 1 µg of 1 Kb plus DNA Ladder (Invitrogen). A UV illuminator with camera was then used to visualize and photograph the gel.

2.2.1.2- Polymerase chain reaction (PCR)

The following standard PCR reaction was assembled (See paragraph: 2.2.1.5 for primer sequence): 1 µg of DNA, 50 µl of Taq PCR Master-Mix (Qiagen), 5 µM of forward and reverse primer and nuclease-free water was added to a final volume of 100µl.

Standard PCR cycle parameters

Using a Thermocycler (MJ Research PTC-200 Pelletier thermal cycler-200), the following standard PCR cycle was used:

[1] 94°C for 1min

[2] 30 cycles of:

(i) 94°C for 1 min (denaturating temperature)

(ii) 60.5°C for 1 min (annealing temperature)

(iii) 72 °C for 1 min (extension temperature)

[3] 72 °C for 10 mins

2.2.1.3- DNA digestion by restriction endonucleases

1µg of plasmid DNA was diluted in 10X enzyme buffer (NEB: New England BioLab) to a final 1X concentration with nuclease-free water (Sigma). This reaction was then

incubated at 37°C for 1-2 hours. Digestion products were loaded on to a 1% agarose gel and extracted using a 'Gel Extraction Kit' (Qiagen), or purified directly using a 'PCR purification kit' (Qiagen).

2.2.1.4- Plasmid ligation, transformation and purification

Linearised vector DNA was added to insert DNA at different molar ratios: 1:1; 1:3; 1:6 to give a final volume of 5 µl. 5 µl of 2X quick ligation buffer (NEB) and T4 DNA quick ligase was added to the reaction mix. This reaction was then incubated for 10 min at RT and then used immediately for transformation or left over-night on ice for next day transformation.

When transforming sub-cloned fragments, NovaBlue Competent Cells (Novagen) were used. Cloning was performed according to the manufacturer recommendations.

Qiagen Mini Prep kits and Qiagen Maxi Prep kits (Qiagen) were used to extract and purify plasmid DNA from single bacterial clones that were grown in LB supplemented with appropriate antibiotics, as per manufacturer's instructions.

2.2.1.5- Cloning of biosyn

The BAP sequence GLNDIFEAQKIEWHE was obtained by annealing the following two oligonucleotides, containing a 5'AgeI and a 3' XbaI restiction site to enable directional cloning.

Forward oligonucleotide (AgeI):
5'- CC GGT GGC CTG AAC GAT ATT TTC GAA GCT CAG AAA ATC GAA TGG CAC GAA GGC TCT TAA T - 3'
Reverse oligonucleotide (XbaI):
5'- CT AGA TTA AGA GCC TTC GTG CCA TTC GAT TTT CTG AGC TTC GAA AAT ATC GTT CAG GCC A - 3'.

The superecliptic pHluorin-synaptobrevin-2 (synaptopHluorin) cloned in a pCI plasmid was used as a template. This construct was provided by Dr G. Miesenböck (Miesenbock et al., 1998).The resulting PCR product was subcloned into the AgeI and XbaI sites of VAMP2-linker-pHluorin, replacing the pHluorin. The construct obtained consists of the

BAP sequence fused to the C terminus of VAMP2 separated by a linker. We refer to this construct as VAMP2-BAP.

The pcDNA3-sec-BirA plasmid is a gift from Professor O.Burrone (International Center for Genetic Engineering and Biotechnology, Trieste, Italy) (Predonzani et al., 2008). It consists of the biotin ligase enzyme, BirA (GenBank: P06709) to which a secretory signal sequence was inserted at its N terminal.

2.2.2- Cultures and transfections

2.2.2.1- Cell line culture

CHO cells are maintained in DMEM medium containing 10% FBS and 1% P/S. There are incubated at 37°C/ 5% CO_2.

2.2.2.2- Cell line transfection

CHO cells were transfected using Lipofectamine. Before transfection, cells were washed once with DMEM medium. They were then left in 2ml DMEM only and serum starved for 1 h. For each plate, 2.5 µg of plasmid DNA was diluted in 200µl DMEM media and 5 µl of Lipofectamine. After mixing by inversion, the complex was left to form for 20 min at room temperature. The mix was then added to the CHO cells. Cells were incubated for 4 h with the DNA/Lipofectamine before being replaced into DMEM/ 10% FBS/1% P/S for continued incubation.

2.2.2.3- Hippocampal cultures

Primary hippocampal cultures were prepared from embryonic day 18 Sprague-Dawley rats of either sex. Hippocampi were dissected out in cold sterile HBSS. Hippocampi were then incubated in 2 ml of 0.5 mg/ml Worthington trypsin, diluted in HBSS, for 15 min at 37°C. In order to stop the trypsin reaction, as much as possible of the trypsin solution was removed, the hippocampi washed three times with HBSS before being replaced with 4 ml of neurobasal media supplemented with 2% FCS and 1% glutamax. For dissociation, cells were gently triturated with fire polished Pasteur pipette and cells number was determined using a haemocytometer. Glass coverslips were previously prepared with 50 µg/ml poly-D-lysine for 1 h at 37°C, washed thoroughly three times with PBS (Sigma) and coated with 20 µg/ml laminin for 1 h at 37°C. Hippocampal cells were plated at 350 cell/mm2 onto the glass coverslips in attachement medium. Neurons

were incubated at 37°C, 6% CO_2. After 2-3 days culture, the attachment medium was replaced with serum-free maintenance medium; this ensured limited glial cell growth. After 3-6 days in culture, neurons were transfected using Lipofectamine 2000.

2.2.2.4- Transfection of hippocampal neurons

Hippocampal neurons were transfected using Lipofectamine 2000. Before transfection, half the medium was removed and replaced with new serum-free medium; the conditioned medium being kept and sterile filtered. The neurons were then left for at least 30 min to recover. For each well, 1µg of plasmid DNA was diluted in Opti-MEM media to a total volume of 25 µl. In addition 1 µl per well of Lipofectamin 2000 was diluted in Opti-MEM media to a total volume of 25 µl and incubated at room temperature for 5 min. The DNA solution and the Lipofectamnie 2000 solution were mixed and incubated at room temperature for 20 min, after what 50 µl of the mix was added to each well of neurons. Neurons were incubated for 16 min with DNA/Lipofectamine 2000 before being replaced into conditioned media for continued incubation. The transfection efficiency of lipofection was approximately 5 -10%.

2.2.3- Labeling Biosyn in CHO cells

CHO cells were transfected with VAMP2-BAP, BirA and GFP plasmids. Immediately following transfection, 100 µM of biotin was added to the medium. CHO cells were initially washed in PBS to remove excess biotin and incubated for 10 min with strep555 to label biotinylated VAMP2. Cells were then rinsed in PBS and fixed. The fixation procedure consist of 30 min incubation with 4%PFA/0.33M sucrose. After fixation, cells were washed three times with PBS before being mounted in mowiol.

2.2.4- Labeling and imaging vesicles with Biosyn

HBS, depolarizing solution and spontaneous solution were all supplement with 1 µM TTX, 25 µM APV and 20 µM CNQX before use.

Primary hippocampal neurons were co-transfected with VAMP2-BAP and BirA plasmids at 3-7 DIV *in vitro*. Immediately following transfection, 100 µM of biotin was added to the medium. Experiments were either carried out 1 week later to ensure that synapses had reached maturity or the following day when studying immature neurons. Experiments were performed in HBS. Neurons were initially washed in HBS to remove excess biotin and incubated for 30 s in strep647 or in unlabelled streptavidin to label all of the biotinylated VAMP2 that was present at the surface of the plasma membrane. To

label the activity-dependent vesicles, neurons were washed three times with HBS to remove excess strep647 or unlabelled strep and then depolarized twice for 90 s by incubation in the depolarizing solution strep555. Each depolarization was separated by a 5 min period. After this treatment, the last depolarization was followed by a 5 min rest before fixation. To label vesicles cycling spontaneously, neurons were washed three times with the spontaneous solution and then incubated 15 min at 37°C in the presence of strep555. Cells were then rinsed in HBS and fixed. The fixation procedure consist of 30 min incubation with 4%PFA/0.33M sucrose. After fixation, neurons were washed three times with PBS before being mounted in mowiol.

Images were obtained either with a Zeiss LSM 510 confocal microscope, a FV1000 Olympus microscope or an inverted microscope Olympus IX71 using a CCD camera (CoolSNAP HQ) controlled by Slidebook software (Intelligent Imaging Innovations). Synapses stained with either streptavidin or antibodies tagged with AlexaFluor 488, 555 and 647 were imaged on the confocal microscope using the correct filter sets. Experiments using the wide-field imaging setup (Olympus IX71) used cells stained with AlexaFluor 488 and AlexaFluor 594 and the excitation (470 ± 15 nm band pass and 565 ± 22nm band pass) dichroic (515 ± 20 nm band pass and 590 nm long pass) and emission (510 ± 15nm band pass and 650 ± 36 nm ban pass) filters. Excitation and emission filters were alternated using a filter wheel. When using confocal microscopy, images were scanned sequentially with each laser to avoid crosstalk.

2.2.5- FM4-64 staining and imaging

Neurons were first washed in HBS. They were then depolarized for 90s with the depolarizing solution containing 1 µM TTX, 25 µM APV, 20 µM CNQX and 10 µM FM4-64 to load synaptic boutons. Finally, neurons were washed with HBS for 10min to remove excess FM4-64 and fixed for 30min with %PFA/0.33M sucrose. After fixation, neurons were washed three times with PBS before being mounted in mowiol.

Images of hippocampal cultures were acquired using Slidebook software (Intelligent Imaging Innovations) and a CCD camera mounted on an Olympus IX71 inverted microscope with an oil lens (100X, 1.4 NA; Olympus). The filter set used was 565 ± 22 nm bandpass exciter, 590 nm long-pass dichroic and 650 ± 36 nm bandpass emission.

2.2.6- Imaging synaptophysin-pHluorin responses

Experiments were carried out as described previously (Li and Murthy, 2001). Coverslips were mounted in a custom-made chamber that was equipped with a pair of parallel platinum electrodes ~5 mm apart, in HBS containing 25 µM APV, 20 µM CNQX. Neurons were stimulated by delivering a 1 ms, 25 mA current pulses using an SD9 stimulator (Grass Instruments), whose timing was controlled by a TTL signal from the imaging software (Slidebook). Bafilomycin A was used at a final concentration of 1 µM. Images were obtained using an inverted microscope, Olympus IX71 with a CCD camera (CoolSNAP HQ) controlled by Slidebok software (Intelligent Imaging Inovations). The light source was a xenon-arc lamp (Lambda LS, Sutter), in which light exposure was regulated by a rapid shutter (Sutter smartShutter) controlled by a Sutter Instruments lambda 10-3 controller, fitted with a 470 ± 20 nm bandpass excitation filter (Chroma Tech) and suitable neutral density filters. Experiments looking at spontaneous vesicle fusion were performed in a humidified, temperature-controlled chamber set at 37°C, in a Ca^{2+} free HBS containing the following (in µM): 1 TTX, 25 APV and 20 CNQX. Images were collected using a Nikon AIR confocal microscope using a 488 nm excitation laser and the appropriate emission filters. Long-term imaging experiments made use of the dynamic auto focus unit to correct for any focus drift during the long periods of acquisition.

2.2.7- Electron microscopy

The electron microscopy project was done in collaboration with the Centre for Ultrastructural Imaging (CUI) directed by Dr Alice Warley with whom I worked closely. The preparation of the samples was done by Mr Brady Ken to whom I am very grateful. I acquired all the pictures presented in this thesis.

DIV 14 hippocampal neurons expressing biosyn were incubated for 15 min at 37°C in HBS containing streptavidin conjugated to AlexaFluor488 and 1.4 nm gold (FluoroNanagold-streptavidin-Alexa488; Nanoprobes), APV, CNQX, TTX and in the absence of Ca^{2+} to label the spontaneous pool of vesicles. Coverslips were rinsed and fixed for 4 hours with 2.5% glutaraldehyde in 0.1M sodium phosphate buffer, pH 7.35 at 4°C. After washing the cells 4 x 10 min enhancement conditioning solution, coverslips were silver enhanced (Silver enhancement kit from Aurion) for 1 h at room temperature (RT). They were then rinsed 2 x 10 min in enhancement conditioning solution, 4 x 5min in phosphate buffer and incubated in 0.5% O_SO_4 (osmium) in 0.1M

buffer for 15 min at RT. Osmium aids to hold to lipids in place. Specimens were then dehydrated in ethanol, and embedded in an araldite resin for 24 hours. Sections (70nm) were post-stained with Uranyl Acetate and Lead Citrate and viewed with the Hitachi 7600 Transmission Electron Microscope or with the FEI Tecnai T12 TEM.

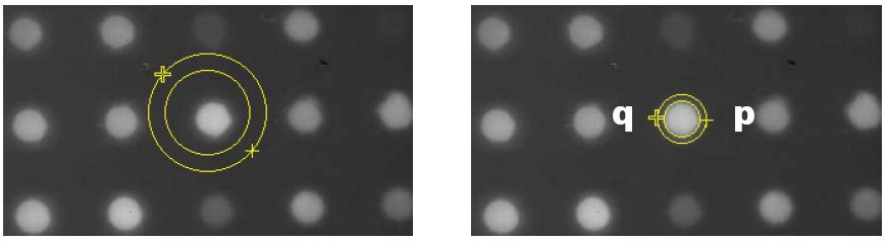
2.2.8- Data analysis

2.2.8.1- Measuring fluorescence intensity

Images were exported as TIFF files and were analyzed using custom-written routines in MATLAB (MathWorks) (Li et al., 2005). Square regions of interest (ROIs, 8X8 pixels in width) were drawn around synapses (generally 4-6 pixels in diameter) and the average fluorescence intensity for each synapse was calculated at its best focal point. When analyzing data from boutons stained for both spontaneous and evoked fusion events, ROIs were selected in the channel used for labeling evoked events and the same ROI coordinates were then applied to other channels. In this way we made sure to take only synapses that had activity-dependent labeling.

2.2.8.2- Measuring localisation of spontaneous and evoked pool of vesicles (cf. Chapter IV, paragraph 4.7)

To measure the localization of spontaneous versus evoked pools of vesicles, the Snakuscule plugin for Image J was used ((Thevenaz and Unser, 2008), http://bigwww.epfl.ch/thevenaz/snakuscule/). It consists in making an active contour around the area of interest based on the fluorescence intensity (see example pictures below). The snakuscule is then defined by the coordinates of two crosses shown as q and p (see figure 2.1).



Images taken from the Biomedical Imaging Group (EPFL, Lausanne, Switzerland) webpage.

Figure 2.1: Snakuscule principle. A broad area of interest ("Snakuscule") is chosen by the user (left panel). After a series of iterative calculations, the Snakuscule converges towards the object until it surrounds it (right panel). The coordinates (q and p) of the snackuscule are then retrieved.

Example of calculation defining the distance separation the spontaneous pool labeled in red and the presynaptic terminal marked by sypHy (green):
q (x_0;y_0); p (x_1,y_1)

The coordinates of the object centre is then calculated for each channels as followed (see Fig. 2.2a):

$x = x_0 + ((x_1-x_0)/2)$

$y = y_0 + ((y_1-y_o)/2)$

The distance d (see Fig 2.2,b) is calculated as follow:

$d_x = |sypHy_x – strep_x|$

$d_y = |sypHy_y - strep_y|$

Pythagoras' theorem is then applied to calculate the distance d:

$d^2 = d_x^2 + d_y^2$

$d = \sqrt{(d_x^2 + d_y^2)}$

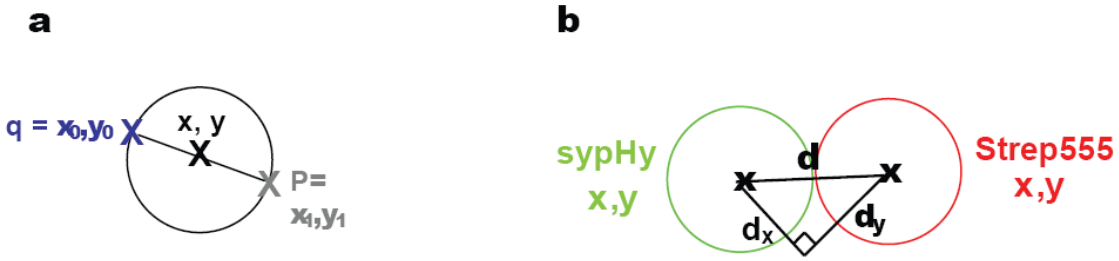


Figure 2.2: Schematic diagram showing the calculation methodology applied to define the distance of the centre between sypHy and strep555 (for spontaneous release). (**a**) Defining the coordinates of the centre of the circle X. (**b**) Pythagoras' theorem applies to calculate the distance d.

2.2.8.3- Measuring the change in fluorescence intensity of the C domain versus the filopodia at the growth cone

Growth cones from 4 to 7 DIV hippocampal neurons expressing sypHy were imaged every 15s for 25 min at 37°C in HBS minus Ca^{2+}, in the presence of TTX Bafilomycin was added after 5 min of baseline imaging. Bafilomycin is a specific proton pump blocker that prevents the re-acidification of vesicles after exocytosis resulting in a cumulative increase in sypHy fluorescence.

In order to compare the change in sypHy fluorescence in the C domain versus the filopodia, a custom-written plugin in Fiji was used. The plugin was written by Olivier Burri, an engineer at the BioImaging and Optics Platform (BIOP) at the EPFL, Lausanne.

The plugin works as follows (Fig. 2.3a):

- First, a maximum intensity projection was done of the time lapse imaging.
- A region of interest (ROI of 69 pixels) is then drawn with the aim of defining the C domain and the filopodia that will be taken into account when measuring the average fluorescence intensity of sypHy over time.
- The area of interest is then straightened over time and a maximum intensity projection was obtained.
- A threshold is then set on the straightened maximum intensity projection to define the C domain
- Once the C domain is defined, it is then transformed into a mask and it is within that mask that the average fluorescence intensity of sypHy will be measured over time.
- To find the filopodia, a threshold is set on the same straightened maximum intensity projection as for defining the C domain, which is then converted into a filopodia mask.
- In order to only measure the average fluorescence intensity of sypHy in the filopodia, the C domain mask was substracted to the filopodia mask.
- Filopodia are highly dynamic therefore the filopodia mask will be different over time (see example Fig. 2.3b).

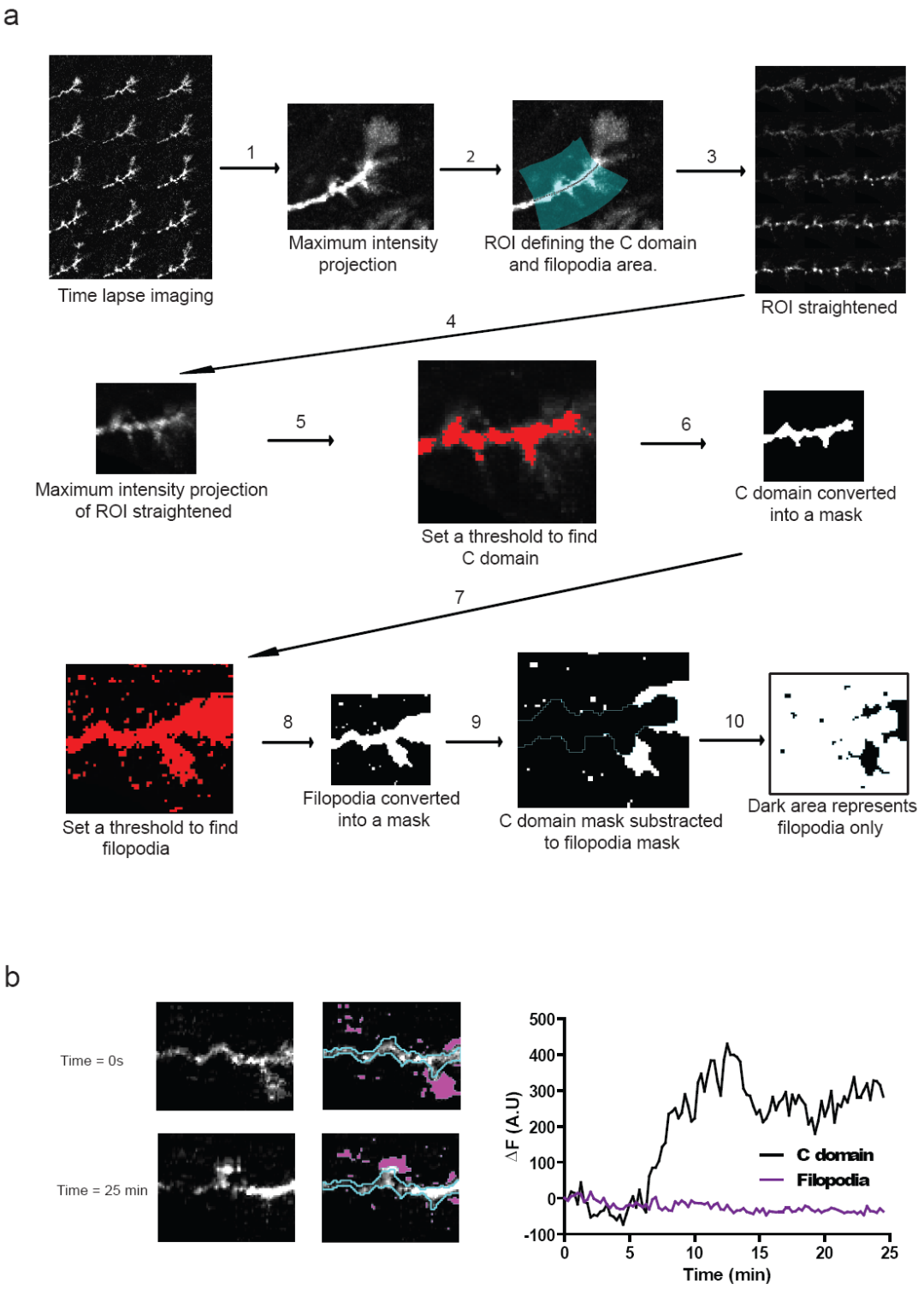


Figure 2.3: Measuring the change in fluorescence intensity of the C domain versus the filopodia at the growth cone. (**a**) Descriptions of the Fiji plugin steps used to measure sypHy average fluorescence intensity at the growth cone C domain and in the filopodia over time. See text for comments (**b**) Example growth cone showing the C domain (encircled in blue) and the filopodia area (represented in purple). The graph on the left represents the average change in sypHy fluorescence over time for both the C domain (black line) and the filopodia (purple line).

2.2.8.4- Measuring the vesicle size

To measure the size of vesicles from EM pictures, the software `reconstruct' (www.synapses.bu.edu) was used. All vesicles contained in the presynaptic terminal were picked by eye and processed for further analysis.

2.2.8.5- Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Statistical comparisons were made by using either ANOVA or t test when appropriate. Significance level was set to 0.05 (95% confidence intervals). To test whether values came from a Gaussian distribution, Kolmogorov-Smirnov and D'Agostino and Pearson's omnibus normality tests were used. If the samples followed a Gaussian distribution, we used the appropriate parametric tests. When this assumption was not met, a nonparametric test was used. Descriptive statistics are reported as mean ± s.e.m throughout the entire thesis.

CHAPTER III

Investigating vesicle cycling at the presynaptic terminal using Biosyn

3.1- Introduction

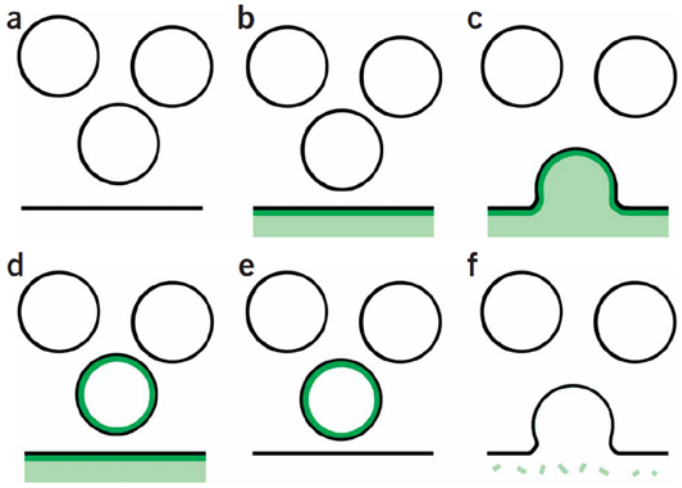
Vesicle cycling at the presynaptic terminal is a key aspect of synapse function and has been studied using different complementary approaches. At the structural level, antibodies have been used to establish the subcellular localisation of proteins that play an important role in neurotransmitter release. The spatiotemporal distribution of these proteins was further explored using fusions of synaptic proteins with green fluorescent protein (GFP) to follow molecules in space and time. These morphological approaches were responsible for a major progress in our understanding of the molecular organisation of the presynaptic terminal.

The next challenge was to explore the function of the presynaptic apparatus. How do synaptic vesicles recycle within a presynaptic bouton? One early approach to measuring vesicle exocytosis and endocytosis in cells and neurons made use of electrophysiological techniques. Electrical measurements of membrane capacitance in single cells or large presynaptic terminals were used to report changes in membrane surface area reflecting the fusion (exocytosis) and retrieval (endocytosis) of synaptic vesicles (Neher and Marty, 1982; Matthews, 1996). This method exploited its high temporal resolution to provide an accurate description of the process of exo and endocytosis in neuroendocrine cells and neurons. Although clearly a useful tool, an important drawback is that it can only be applied to synaptic preparations in which the presynaptic terminal is large enough that it can be directly patched and where a large number of small synaptic vesicles fuse with the plasma membrane. It is thus restricted to just a few preparations with particularly large presynaptic terminals, such as goldfish retinal bipolar neurons, frog hair cells, the calyx of held and the large mossy fiber terminals originating from dentate gyrus granule cells (see for review: (Royle and Lagnado, 2003)). Although capacitance measurements provide high temporal resolution it cannot be used to study the fusion of a single small synaptic vesicle, as this would be below the level of resolution for this technique. In preparations where it has been used, capacitance measurements have provided important information about presynaptic

mechanisms, such as the rate of exo- and endocytosis and the relative sizes of distinct vesicle pool (Royle and Lagnado, 2003). Unfortunately, as with most electrophysiological approaches, it does not give any insight into the localisation and origin of these events. For this purpose, live imaging methods have been developed to study synaptic vesicle exo- and endocytosis in central and peripheral nerve preparations.

One of the first approaches made use of a fluorescently-tagged antibody directed against the luminal domain of synaptotagmin 1 (Syt1-Ab), an important synaptic vesicle protein acting as a Ca^{2+} sensor for neurotransmitter release (Matteoli et al., 1992). This method allowed the specific labelling of synaptic vesicles after they had undergone fusion with the plasma membrane. It could be used to monitor vesicle traffic over a long period of time (minutes to hours) because of the stable association between the antibody and the antigen (Kraszewski et al., 1995). However, the relatively slow binding kinetics of the fluorescently-labelled Syt1-Ab precludes accurate measurements of neurotransmitter release kinetics (Kraszewski et al., 1995).

An important advance in the field of presynaptic function arose with the development of novel fluorescent styryl dyes (FM1-43, FM4-64 and FM2-10) (Betz and Bewick, 1992). These lipophilic dyes bind with fast ‘on’ (in the millisecond range) and relatively fast ‘off’ (in the seconds range) rates to the plasma membrane and become fluorescent when attached to membrane phospholipids. During stimulation, FM dyes will be taken up by vesicles fusing with the plasma membrane after re-endocytosis. Importantly, these dyes do not cross the lipid bilayer and can therefore be used for the prolonged labelling of vesicles. Nonetheless, they will diffuse out of the vesicles if they undergo a new round of exocytosis. Consequently, a stimulation of preloaded nerve terminals after the dye has been removed from the extracellular medium will result in a decay of bouton fluorescence. This dye unloading can serve either as a direct measure of the rate of exocytosis or as an indirect assessment of the amount of endocytosis that occurred during the dye loading period. This widely used method has enabled quantitative measurements of both exo- and endocytosis in small synaptic boutons, with good spatial resolution and has provided significant advances in our comprehension of synaptic vesicle dynamics and organisation (Fig. 3.1(Gaffield and Betz, 2006). However, as FM dyes also stain membrane compartments unrelated to synaptic activity, care must be taken when interpreting the data.

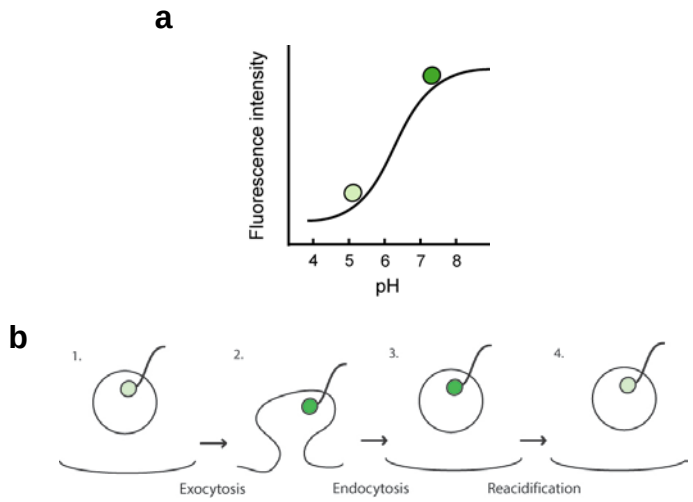


Gaffield, M.A., and W.J. Betz. 2006. *Nat Protoc*.

Figure 3.1: Schematic diagrams showing how FM dyes are used to study vesicle cycling. (**a**) Synaptic vesicles near the plasma membrane. (**b**) FM dye is added, binds to the outer membrane, and becomes fluorescent. (**c**) The preparation is stimulated and a vesicle fuses with the plasma membrane exposing the luminal membrane to the FM dye. (**d**) The vesicle is endocytosed with FM dye inside. (**e**) The FM dye is washed out and a labelled vesicle is imaged. (**f**) The preparation is stimulated again in a dye-free medium and vesicle exocytosis is measured as dye leaves the fusing vesicle.

A more recent development has overcome some of these drawbacks in the form of new genetically-encoded probes that report presynaptic vesicle cycling in real time. It is based on the fusion of a pH-sensitive GFP called pHluorin to the luminal domains of presynaptic proteins (e.g. VAMP2-pHluorin or synaptopHluorin, (Miesenbock et al., 1998) synaptophysin-pHluorin (Granseth et al., 2006), synaptotagmin-pHluorin (Wienisch and Klingauf, 2006) and vGlut-pHluorin (Balaji and Ryan, 2007)). This tool specifically labels presynaptic boutons and reports changes in fluorescence following exo- and endocytosis. The lumen of presynaptic vesicles is acidic (pH 5.5) and at this pH the fluorescence of the pHluorin is quenched. When vesicles fuse with the plasma membrane they expose their intraluminal proteins to the more basic extracellular medium (pH 7.3), which causes a rise in pHluorin fluorescence. Following endocytosis, vesicles re-acidify and pHluorin fluorescence decreases (Fig. 3.2 (Burrone et al., 2006)). One of the advantages of this technique is its good spatial resolution that enables to monitor the function of individual presynaptic terminals. However, pHluorin has two main disadvantages. First it does not allow the labelling of functional subsets of vesicles as the genetic construct used is expressed equally in all vesicles. Second, the presence of an important fraction of pHluorin at the surface of the presynaptic terminal causes a

decrease in sensitivity, making it difficult to detect small synaptic events (Sankaranarayanan et al., 2000; Granseth et al., 2006).



Burrone, J., Z. Li, and V.N. Murthy. 2006. *Nat Protoc.*

Figure 3.2: Schematic diagrams showing the principle of pHluorin. (**a**) Graph showing the change in pHluorin fluorescence as a function of the pH. (**b**) (1) At rest, pHluorin fluorescence is quenched as the vesicle intralumen is at pH~5.5. (2) During exocytosis, vesicles fuse with the plasma membrane and expose their lumen to the basic extracellular medium (pH 7.3) causing a rise in pHluorin fluorescence. (3) and (4) Following endocytosis and reacidification, pHluorin returns to its quenched state.

We have developed a novel technique to specifically label presynaptic vesicle proteins in live neurons based on the well-established interaction between biotin and avidins. This method involves the specific biotinylation of a biotin acceptor sequence (BAP) fused to the luminal end of the synaptic vesicle protein VAMP2 (synaptobrevin 2). The 15 amino acids-long BAP domain is specifically recognized by the *Escherichia coli* enzyme biotin ligase (BirA), which then covalently attaches a single biotin molecule to a specific lysine residue of the BAP sequence (Predonzani et al., 2008). Furthermore, the BAP motif is not recognized by mammalian enzymes with biotin ligase activity (Howarth et al., 2005), nor does BirA recognize endogenous mammalian biotinylation motifs (de Boer et al., 2003), rendering the biotinylation of BAP domains very specific. Taking advantage of the high affinity between biotin and avidins ($K_d \approx 10^{-15}$ M), fluorescently-tagged streptavidin was used to irreversibly label biotinylated VAMP2. This biotinylation system was exploited to develop a tool that would allow the study of vesicle cycling at presynaptic terminals. The aim of this chapter is to characterize the sensitivity, utility and reliability of our new technique in reporting presynaptic activity.

3.2- Design and expression of biosyn

3.2.1- Design and generation of VAMP2-BAP and BirA expression constructs

The first step in the creation of biotin-tagged vesicles was to genetically fuse the BAP motif to the luminal end (C terminal) of the presynaptic vesicle protein VAMP2 (synaptobrevin 2). The sequence encoding the BAP motif (GLNDIFEAQKIEWHE) was obtained by annealing two oligonucleotides (see Chapter II Material and Methods). As a starting point, a VAMP2-linker-pHluorin construct obtained from G. Miessenbock (Oxford University) was used (Miesenbock et al., 1998). The pHluorin sequence from this construct was removed and replaced by the BAP motif, resulting in a VAMP2-linker-BAP fusion (VAMP2-BAP).

The BirA enzyme expression plasmid consists of an engineered version of the enzyme BirA (secBirA) that contains a secretion signal peptide which leads to its translocation to the endoplasmic reticulum, where secretory proteins containing the BAP motif will be biotinylated (See Chapter II Material and Methods) (Predonzani et al., 2008).

Thus, co-expression of both VAMP2-BAP and BirA results in the luminal biotinylation of VAMP2, a molecule we called ‘biosyn’. Biosyn is then transported along the axon toward the presynaptic terminal where it integrates into the synaptic machinery (Fig. 3.3a). Here, the tight binding that exists between biotin and streptavidin (Kd ~ 10^{-15}) was exploited and fluorescently-tagged streptavidin was used to irreversibly label vesicles containing biosyn. Indeed, our prediction was that when vesicles fuse with the plasma membrane, exposing their intraluminal biotin, they would bind the fluorescently-labeled streptavidin (strep) and subsequently take it up during endocytosis (Fig. 3.3b).

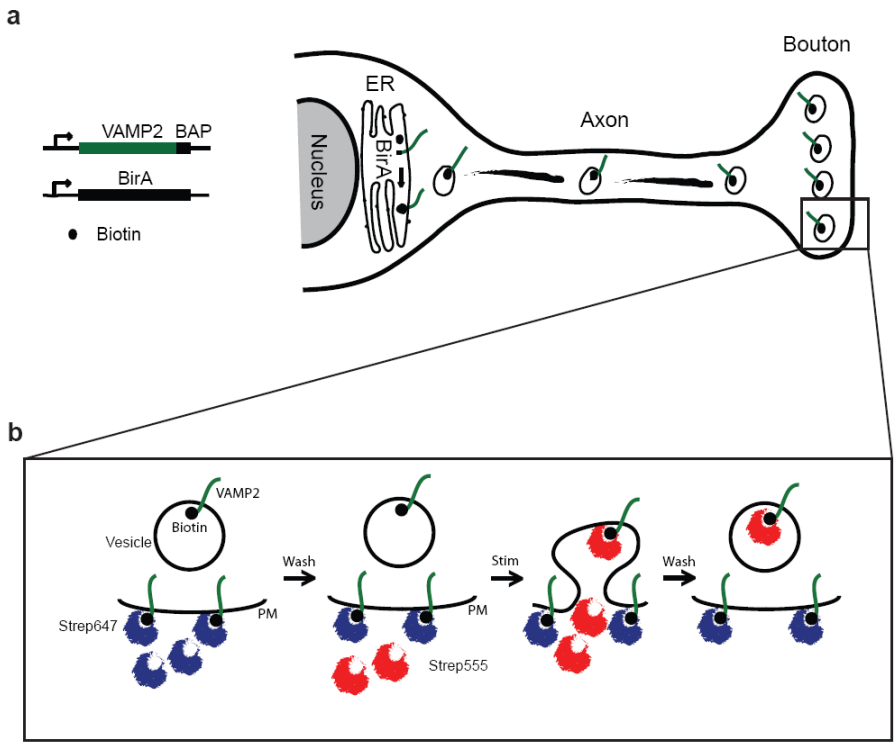


Figure 3.3: Method: Schematic diagrams showing the principle of Biosyn labelling with streptavidin. (a) The method involves the specific biotinylation of a biotin acceptor peptide (BAP) fused to the lumen end of the presynaptic protein VAMP2 (synaptobrevin 2). The BAP domain (15 amino acids) is specifically recognised by the bacterial (E.coli) biotin ligase enzyme BirA. Co-transfection of both VAMP2-BAP and BirA results in the luminal biotinylation of VAMP2. This reaction happens in the endoplasmic reticulum (ER). Biosyn is then transported along the axon towards the presynaptic terminal where it is recruited. (b) Diagram showing labelling of biosyn with streptavidin (strep). Neurons are first pre-incubated with streptavidin Alexa647 (strep647, shown in blue) to quench surface biosyn. Excess strep647 is removed by washing. Neurons are subsequently stimulated in the presence of strep555 (shown in red) to label the vesicles that fuse with the plasma membrane during neurotransmitter release and expose their intraluminal biosyn to the extracellular medium.

3.2.2- Expression in cell lines

Biosyn expression and proof-of-principle of the technique was first tested on cell lines. CHO (Chinese Hamster Ovary) cells were co-transfected with VAMP2-BAP, BirA and EGFP. Biotin was added in excess (100µM) to the culture media to ensure that it would not be a limiting factor in the biotinylation reaction of VAMP2-BAP. The detection of biosyn was done the following day as transient expression is optimal 24 to 48 hours after transfection (Dalby et al., 2004). Transfected cells were initially washed three times with PBS to remove the excess of biotin and then incubated for 5 minutes in PBS solution containing streptavidin 555 (strep555) to label any biosyn present on the

surface of the plasma membrane. This resulted in strong labeling (red, in Fig. 3.4) of EGFP cells also expressing both VAMP2-BAP and BirA (Fig.3.4). Furthermore, no staining was detected in cells expressing only VAMP2-BAP or only BirA. These results indicate that the biotinylation reaction of VAMP2-BAP is specific, has little background, and can be detected in live cells, using fluorescent streptavidin conjugates.

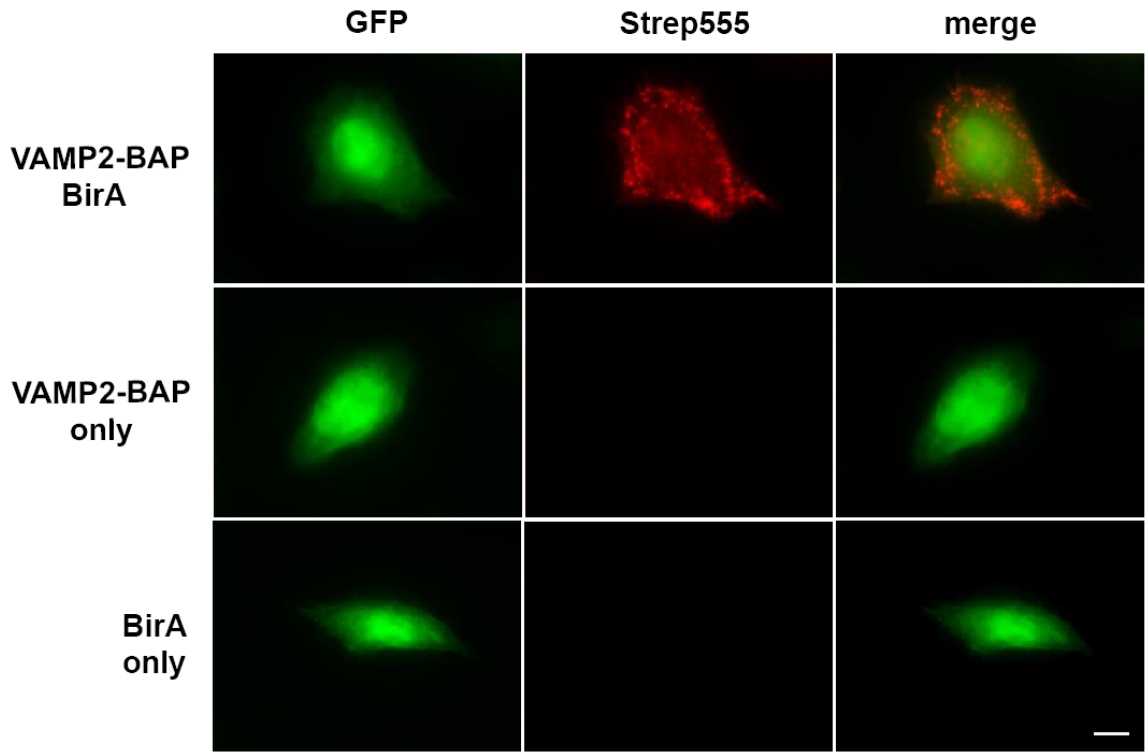
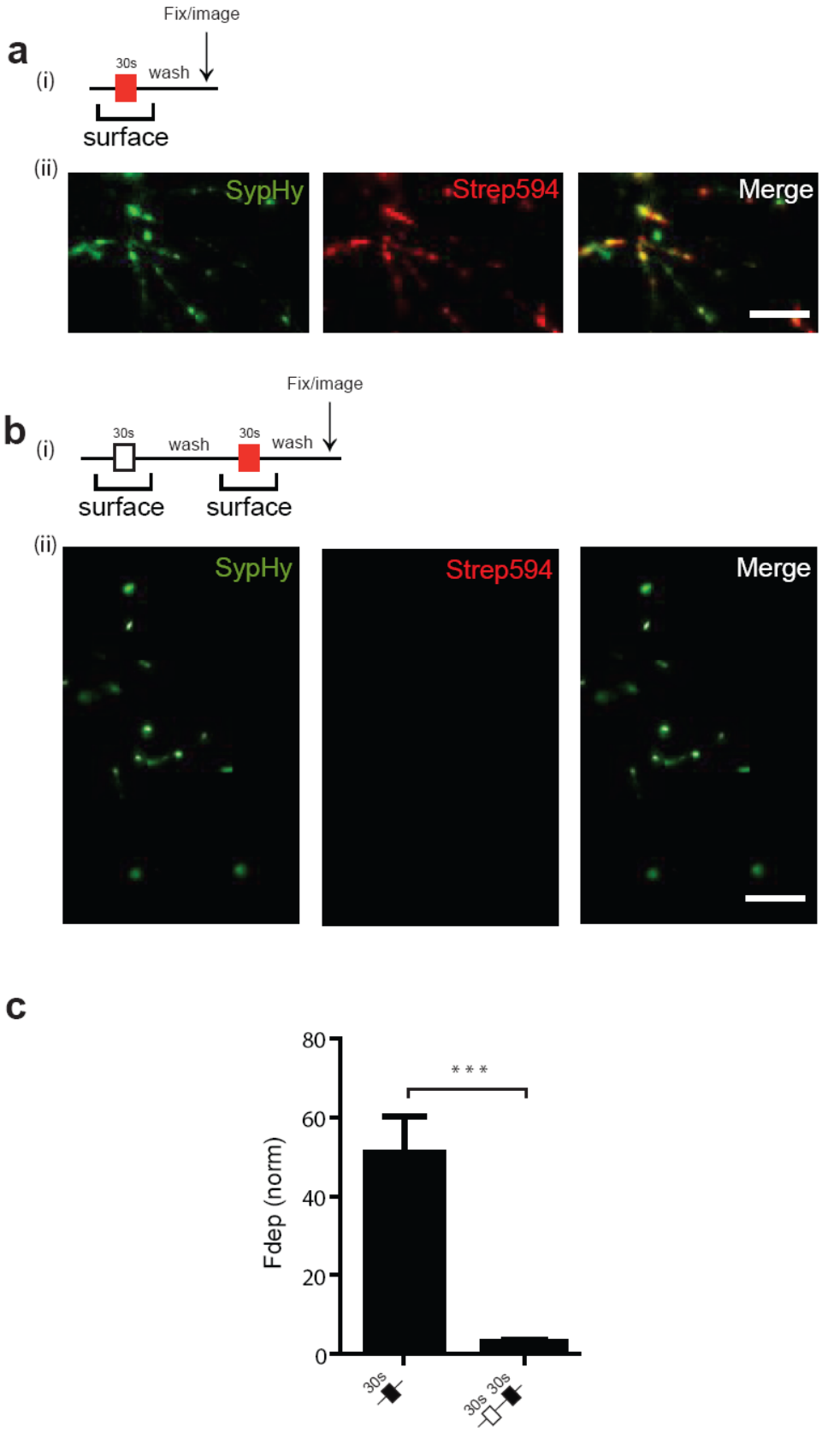


Figure 3.4: Expression of biosyn in CHO cells. CHO cells expressing VAMP2-BAP (V2), BirA (BA) and EGFP showed a strong labelling in red after 5 min exposure to PBS in the presence of strep555 (top). Cells transfected with EGFP and either VAMP2-BAP (middle) or BirA (bottom) alone did not show any staining. Scale bar = 10 µm.

3.2.3- Expression in hippocampal neurons

Previous studies have shown that VAMP2 is not exclusively found in presynaptic vesicles, but is also present on the plasma membrane of presynaptic boutons. Biochemical, as well as fluorescence imaging experiments (Sankaranarayanan et al., 2000; Wienisch and Klingauf, 2006) and immunogold labelling of freeze-fractured neurons (Taubenblatt et al., 1999) showed that around 20 to 30% of presynaptic VAMP2 is located on the presynaptic membrane. This high level of surface expression should therefore easily be detected by streptavidin labelling of surface biosyn at presynaptic terminals. To investigate if this was indeed the case, hippocampal neurons were transfected at DIV 7 with VAMP2-BAP, BirA, and the presynaptic reporter, synaptophysin-pHluorin (sypHy). The latter consists of a fusion protein between synaptophysin and a pH-sensitive enhanced green fluorescent protein (pHluorin) (Granseth et al., 2006), which in this case was used simply as a marker of presynaptic boutons. At day 14, when synapses are mature, neurons expressing biosyn were first washed to remove the excess extracellular biotin and then incubated for 30s in Hepes buffered saline (HBS) containing strep594, to label all surface biosyn. All experiments were done in the presence of tetrodotoxin (TTX), which prevents neurons from firing Na^+-dependent action potentials, and the glutamate receptor antagonists CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and APV (2-amino-5-phosphonovaleric acid) that abolish AMPA and NMDA mediated excitatory synaptic transmission, respectively (see Methods). Figure 3.5a shows that individual synapses expressing sypHy (green) co-localised with surface biosyn labelled with strep594 (red). In order to check that all the surface biosyn was labelled during the 30s incubation with strep594, in another set of experiments, neurons were first incubated for 30s in HBS containing unlabelled streptavidin. After washing away the excess of unlabelled streptavidin, a further 30s incubation in HBS containing strep594 resulted in no labelling (Fig. 3.5b). Quantification of the total fluorescence intensity per bouton showed a drastic reduction in the fluorescence intensity of strep594 when surface biosyn was first exposed to unlabelled streptavidin ($P < 0.0007$) (Fig. 3.5c). These results confirm the presence of a fraction of biosyn at the plasma membrane and that, importantly for future experiments, all of the surface molecules can be fully saturated by pre-incubating the neurons for 30s in the presence of streptavidin.

Figure 3.5: Labelling surface biosyn. (**a**) (**i**) Schematic diagram showing the time line of the experimental protocol. Hippocampal neurons were co-tranfected with biosyn and sypHy. (**ii**)Images show co-localisation of individual synapses expressing sypHy (green) with surface biosyn labelled with strep594 (red). Scale bar = 2 µm (**b**) (**i**) Schematic diagram showing the time line of the experimental protocol. Surface biosyn was stained with unlabelled streptavidin for 30s and washed. Neurons were then exposed for a further 30s with strep594. After washing, neurons were imaged. (**ii**) Images show no labelling of biosyn with strep594. Scale bars = 2 µm. (**c**) Quantification of the fluorescence intensity of surface biosyn labelled with strep594 under different conditions shown below each bar. The fluorescence intensity plotted corresponds to the treatment denoted as red squares. The first bar (from the left) show the intensity of surface biosyn (n= 135 synapses from 5 cells). The second bar show that surface labelling of biosyn (30s exposure to strepNo) saturated all biosyn binding sites (n= 285 synapses from 10 cells). ($P < 0.0007$). Values are shown as mean ± s.e.m.


a
(i)
Fix/image
30s
wash
surface
(ii)
SypHy
Strep594
Merge
b
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wash
surface
surface
(ii)
SypHy
Strep594
Merge
c
Fdep (norm)
80
60
40
20
0
***
30s
30s 30s

3.3- Biosyn labels vesicles that fuse in response to neuronal activity

Having established that a fraction of biosyn is present on the membrane of the presynaptic terminal, the surface biosyn was first saturated before using the probe to follow vesicle exocytosis (Fig. 3.3b). Neurons expressing VAMP2-BAP and BirA were first incubated for 30s in HBS containing strep647 to quench surface biosyn. After washing out the excess of strep647, neurons were depolarized twice by incubating in high potassium solution (65 mM KCl) for 90 seconds (see Chapter II Material and Methods) containing strep555, to ensure that all the newly exocytosed vesicles were labelled. This stimulation protocol should mobilise the entire pool of recycling vesicles. As vesicles fuse with the plasma membrane, they expose their luminal biosyn to the extracellular medium and become labelled with strep555 (Fig. 3.6a). We found that sypHy positive presynaptic terminals (green puncta in Figures 3.6b) showed labelling of both surface biosyn (blue) and biosyn that belonged to presynaptic vesicles mobilised by membrane depolarization (red in Fig. 3.6b). In a control experiment, no staining with strep555 was detected when neurons expressing either VAMP2-BAP or BirA alone were depolarized (Fig. 3.7a,b), which confirms that streptavidin specifically labels axons expressing biosyn.

Extracellular high potassium stimulation depolarizes the plasma membrane and opens voltage-gated calcium channels causing Ca^{2+} influx and neurotransmitter release. To further characterise the specificity of biosyn labelling, neurons were depolarized in conditions that are not conducive to vesicle cycling, such as in the presence of cadmium (a non-selective antagonist of voltage-gated calcium channels), in the absence of Ca^{2+} or in the absence of high potassium. All these treatments resulted in significantly lower fluorescence intensity when compared to high potassium treatment in the presence of Ca^{2+} (Fig. 3.7c).

Taken together, these results demonstrate the specificity of the biosyn technique to label evoked neurotransmitter release at a single synapse.

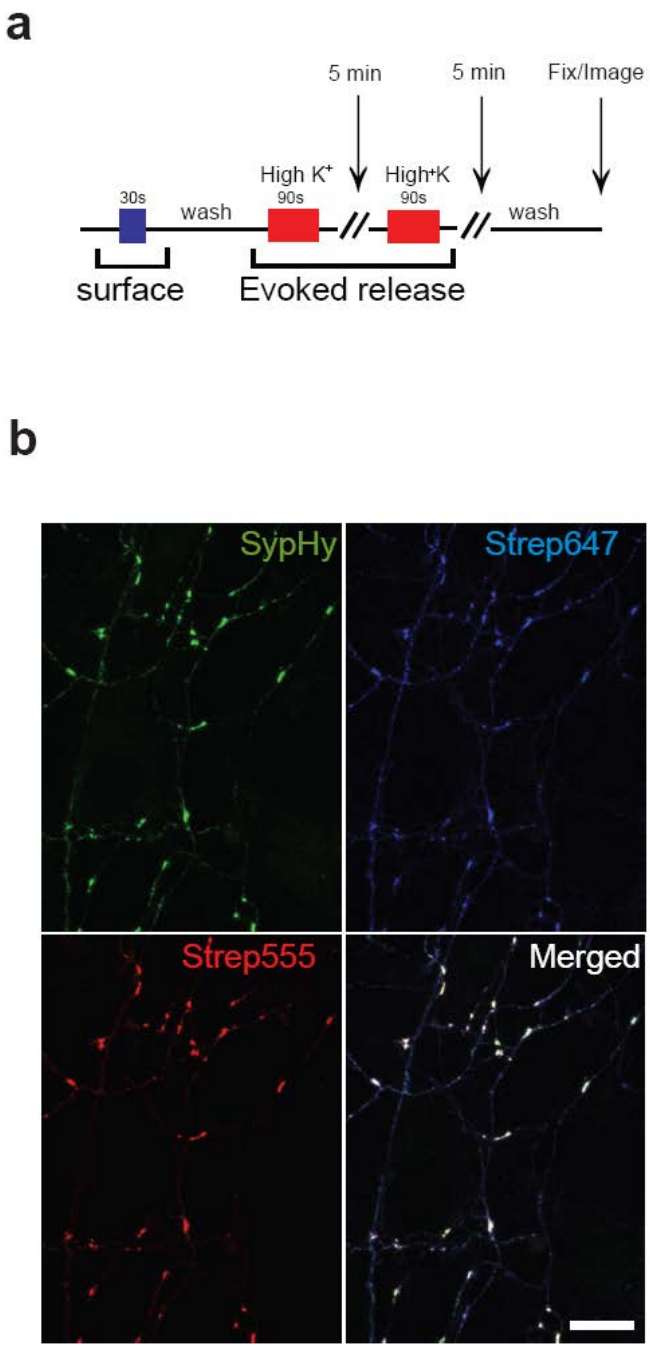


Figure 3.6: Labelling vesicles released by depolarisation. (**a**) Schematic diagram showing the time line of the experimental protocol. Surface biosyn was stained with strep647 (blue) for 30s and washed. Neurons were then stimulated twice with high potassium solution in the presence of strep555 (red). After each depolarisation, 5 min rest was allowed. After washing, neurons were fixed and imaged. (**b**) Hippocampal neurons were co-transfected with sypHy and biosyn. Images show co-localisation of individual synapses expressing sypHy (green) with the recycling pool of vesicle labelled with strep555 (red) and surface labelling with srep647 (blue). Scale bar = 10 µm.

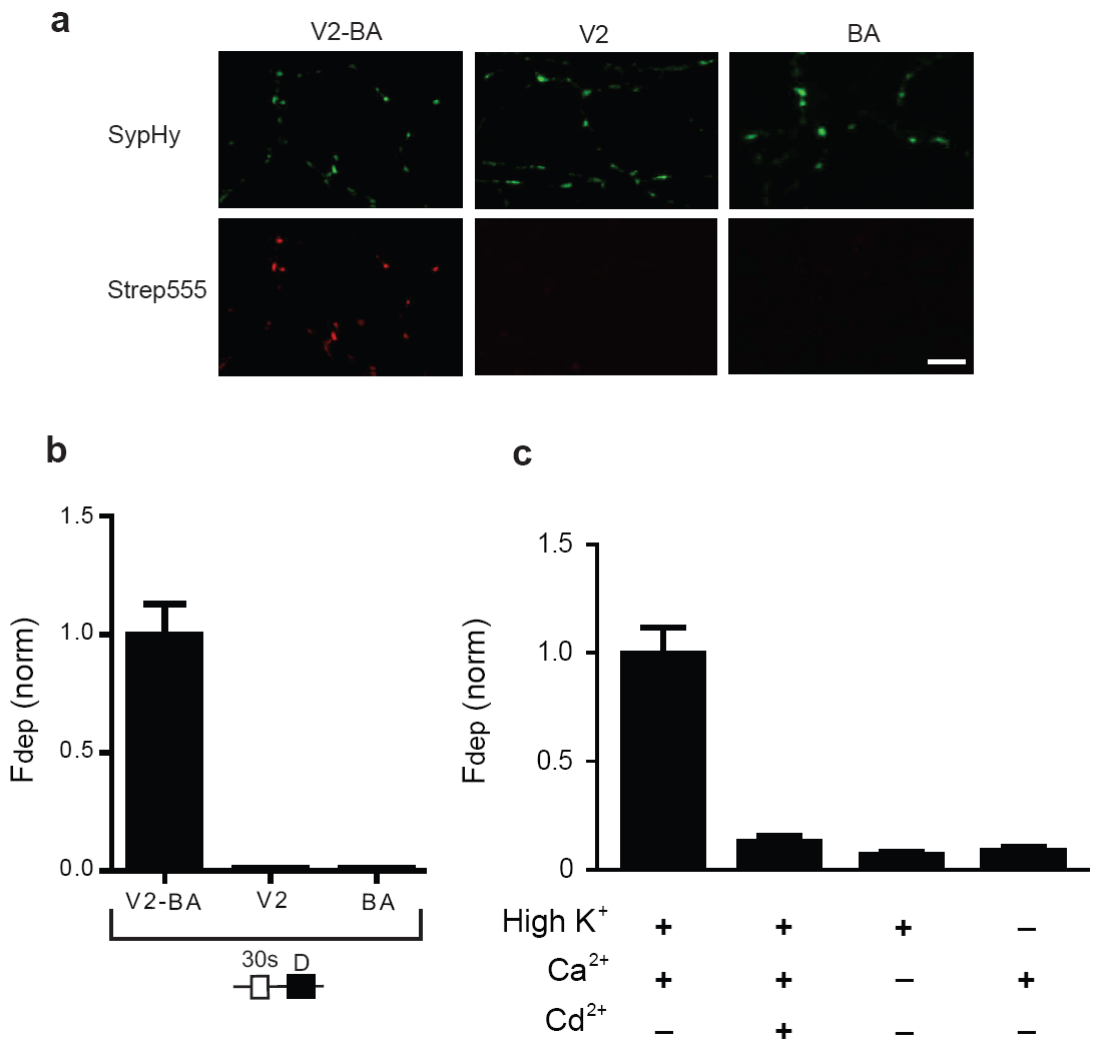


Figure 3.7: Specificity of the biosyn probe. (**a**) Neurons expressing sypHy, Vamp2-BAP (V2) and BirA (BA) showed strong labelling with Strep555 after two consecutive high K^+ stimuli (left). Neurons transfected with sypHy and either VAMP2-BAP (middle) or BirA (right) alone did not show any staining. Scale bar = 5 µm. (**b**) The graph shows the pooled fluorescence intensity data of synapses labelled with a depolarizing stimulus (D) in the presence of strep555. Fluorescence values were normalized to the size of the recycling pool. The schematic below shows the protocol followed. Open bars represent 30 seconds of surface staining with unlabelled streptavidin; closed bars represent staining with strep555. Neurons were transfected in three different ways: with VAMP2-BAP and BirA (V2-BA), with VAMP2-BAP only (V2) and with BirA only (BA). Note that strong labelling is only observed when both VAMP-2 and BirA are co-expressed (V2-BA). When either one is expressed independently (V2 or BA) no labelling was observed after high K^+ stimulation. (**c**) Quantification of biosyn staining under different conditions. High K^+ stimulation specifically labeled the activity-dependent pool when Ca^{2+} was present. All conditions were normalized to this value. Staining was abolished when Ca^{2+} was absent, when cadmium (a calcium channel blocker) was present (n >200 synapses from 10 cells per condition) or when high K was absent. Values are shown as mean ± s.e.m.

Recently, a study by Clayton *et al.* has shown that bulk endocytosis is more likely to occur in response to a strong and/or sustained stimulus (Clayton et al., 2008). This form of endocytosis corresponds to the retrieval of a large area of plasma membrane from which subsequent clathrin-coated synaptic vesicles may bud (Royle and Lagnado, 2003; Clayton and Cousin, 2009). It is thought to function as a compensatory form of endocytosis in response to high levels of membrane fusion during intense stimulation. In order to verify whether bulk endocytosis happens in response to the high potassium stimulation protocol used in this study, ultrastructural analysis of synaptic vesicles was carried out (Fig. 3.8a). Following 90s depolarisation, no vesicles above 50 nm were detected (Fig. 3.8a,b), suggesting that bulk endocytosis did not happen in response to the high potassium stimulation in our system. These results further confirm that the puncta visualised with biosyn correspond to the labelling of small clear vesicles.

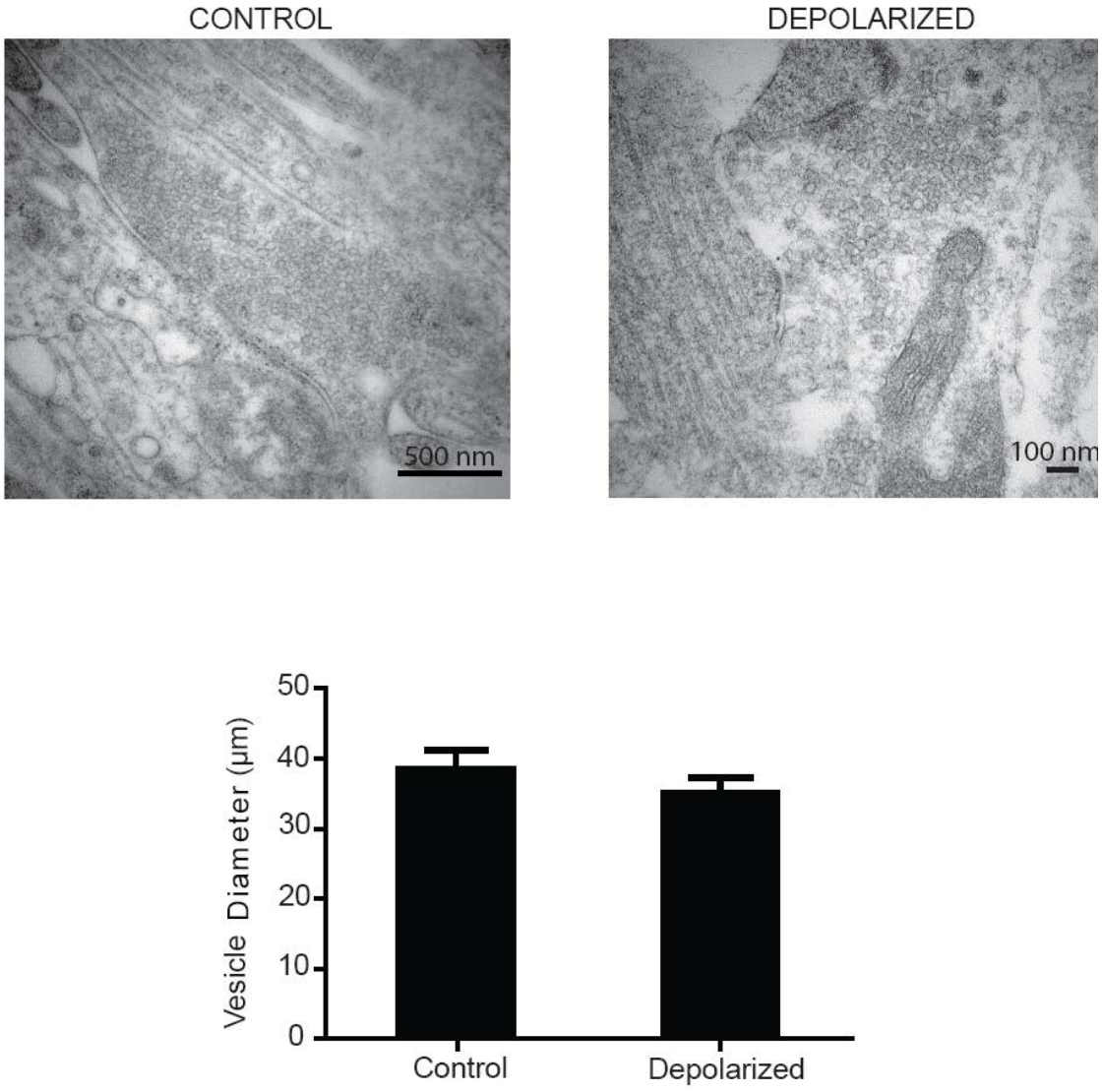


Figure 3.8: High KCl stimulation does not cause bulk endocytosis. (a) Transmitted electron microscopy (TEM) images from untreated synapses (control, on the top left) and depolarized with 65mM KCl (on the top right). (b) The graph represents the average size of the synaptic vesicles of untreated neurons and following 90s depolarization. (n= 6 synapses per condition). Statistical analysis was performed using Mann Whitney test (p= 0.4). Values are shown as mean ± s.e.m.

3.4- Biosyn is a reliable tool to report exocytosis

From the above experiments, biosyn was shown to consistently label vesicles that recycle in response to activity. The next step was to check whether biosyn fluorescence could also be used to report the amount of exocytosis that happens at a single synapse. To establish this we needed an independent measure of vesicle exocytosis at the synapse that would allow direct comparison with biosyn labelling. This was done by combining the expression of biosyn with a sensitive marker for exocytosis, synaptophysin-pHluorin (sypHy). SypHy is a fusion protein between synaptophysin and a pH-sensitive EGFP (pHluorin) that can detect the difference in pH between the acidic environment of the vesicle lumen and the extracellular space. At rest, sypHy fluorescence is quenched, as its pHluorin molecule resides in the acidic lumen of the vesicle (pH ~ 5.5). When a vesicle fuses with the plasma membrane and exposes its proteins to the basic extracellular media (pH ~ 7.3), its fluorescence increases (Granseth et al., 2006). Once the membrane and its corresponding proteins are internalised, the vesicle is reacidified resulting in a decrease in fluorescence back to its original resting state. Although sypHy is a sensitive reporter of vesicle exocytosis, its signal will be contaminated by any ongoing endocytosis that occurs during the stimulus. To exclusively measure exocytosis at presynaptic terminals, the sypHy probe can be combined with the 'alkaline trap' method, which uses an antagonist of the vesicular proton pump (such as bafilomycin) to prevent the re-acidification of the vesicles after exocytosis. This results in a cumulative increase in sypHy fluorescence signal which is proportional to the number of vesicles that have fused with the plasma membrane in response to stimulation (Burrone et al., 2006).

Neurons co-expressing biosyn and sypHy were stimulated with 900 action potentials (APs) at 20 Hz, in the presence of bafilomycin (Fig. 3.9a). This resulted in a large and measurable increase in sypHy fluorescence that remained elevated and did not return back to baseline, due to the presence of bafilomycin (Fig. 3.9b). As illustrated in figure 9c, the gray traces represent the change of sypHy fluorescence for individual synapses and the black ones represents the average of all the synapses for this neuron. The amplitude of the change in fluorescence corresponds to the amount of exocytosis at a given synapse. While measuring the change in sypHy fluorescence, strep594 was present in the extracellular media to label exocytosed vesicles (Fig. 3.9a). Indeed, following stimulation and removal of excess strep594, synapses were strongly labelled with strep594 (red puncta in Fig. 3.9d) and co-localised with sypHy (green puncta in

Fig. 3.9d). Furthermore, a strong correlation was found between the change in sypHy fluorescence (ΔF) and the intensity of strep594 fluorescence at single synapses, in data obtained from a single axon (data not shown) or when pooled from many axons (correlation coefficient $R= 0.81$, $P < 0.05$) (Fig. 3.9e).

To further explore the correlation between the change in sypHy fluorescence and in strep594 fluorescence intensity, neurons were stimulated using a milder, more physiological stimulus. The same experimental protocol as figure 3.9 was applied, but this time the neurons were stimulated with 40 APs at 20 Hz to recruit a small population of vesicles at a presynaptic terminal, which is normally referred to as the rapidly releasable pool (Burrone et al., 2006). This pool represents the vesicles that are preferentially released in response to action potentials. A strong correlation was also found between the change in sypHy fluorescence and strep594 labelling ($R = 0.59$, $P < 0.0001$) (Fig. 3.10).

Taken together, these results show that biosyn can be used as a reliable tool to report exocytosis in pre-synaptic terminals.

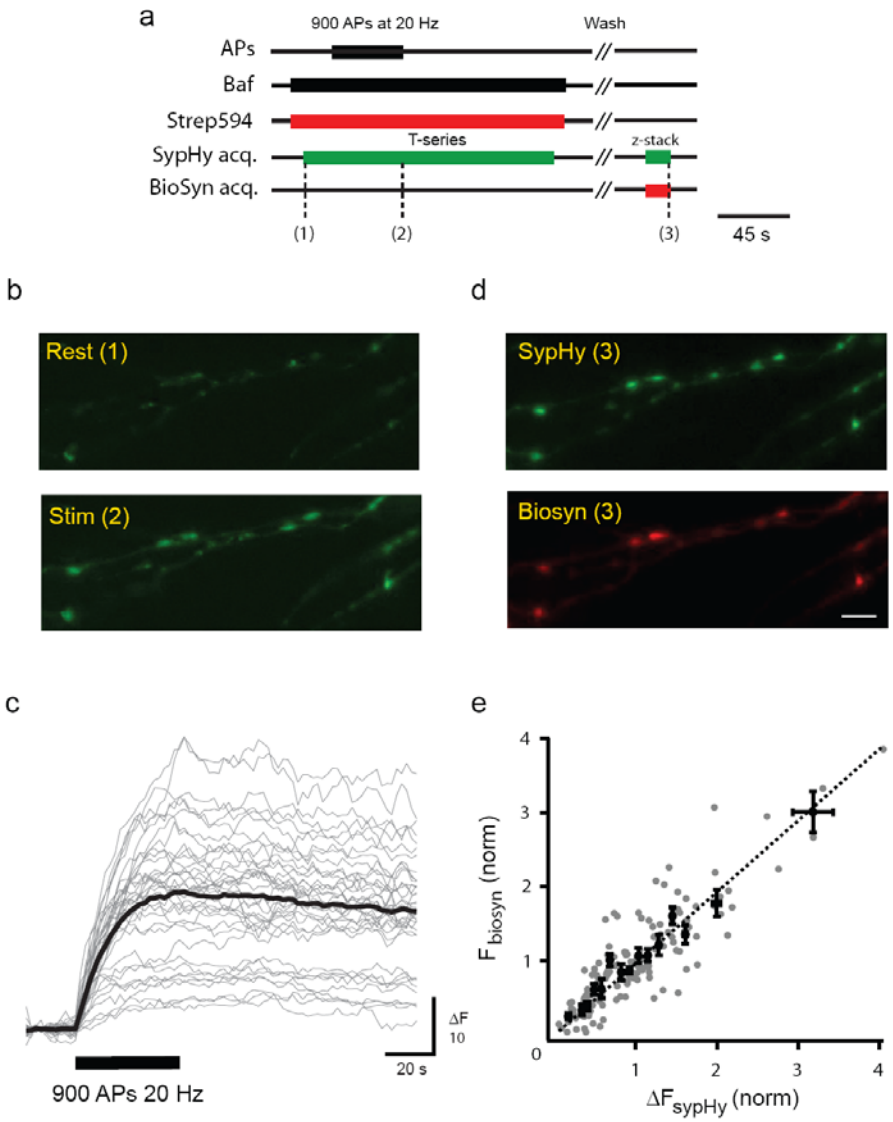


Figure 3.9: Biosyn is a reliable tool to report exocytosis. (a) Schematic diagram showing the outline of the experimental protocol applied on hippocampal neurons expressing biosyn and sypHy. Neurons were stimulated by a train of 900 AP at 20 Hz, in the presence of bafilomycin to measure the amount of exocytosis reported by the change in sypHy fluorescence and strep594 was used to label biosyn. (b) Images show the change in sypHy fluorescence before (Rest 1) and after stimulation (Stim 2). Scale bar = 5µm (c) Traces representing sypHy responses of a single synapse (gray) and the average response (black) to 900 AP at 20Hz. (d) Images show a strong labelling of synapses with strep594 after stimulation which co-localise with sypHy. Scale bar represents 5µm. (e) Graph showing a plot of the biosyn fluorescence intensity as a function of the change in sypHy fluorescence. Individual synapses are shown in gray and the binned (groups of 10) values in black (n = 145 synapses from 6 cells). The black line represents the best linear fit to the data, constrained to go through the origin. (correlation coefficient R= 0.81, $P < 0.05$). Values are shown as mean ± s.e.m.

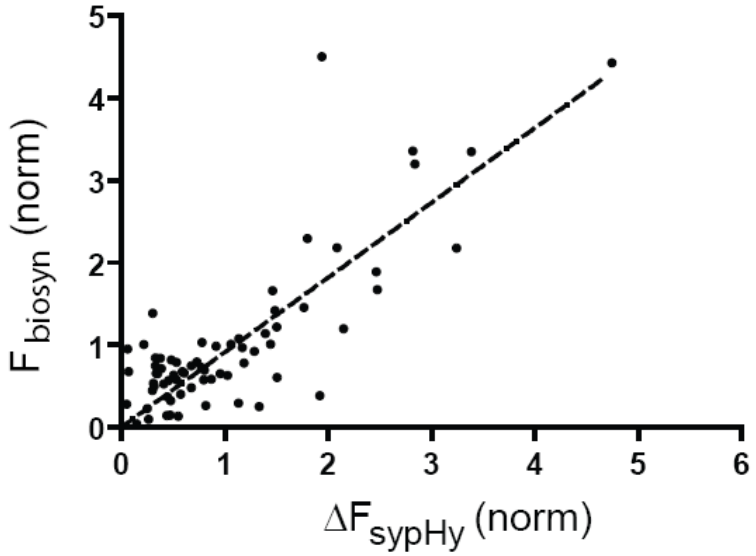


Figure 3.10: Strong correlation found between SypHy and biosyn labelling of the RRP. Neurons expressing biosyn and sypHy were stimulated with a train of 40 AP at 20 Hz. The graph shows a plot of the biosyn fluorescence intensity as a function of the change in sypHy fluorescence (n = 50 synapses from 3 cells). The black line represents the best linear fit to the data, constrained to go through the origin. Correlation coefficient = 0.59, $P < 0.0001$.

3.5- Biosyn labelling does not affect vesicle cycling

Finally, an important control was to verify that vesicle cycling is not affected by the labelling of biosyn with streptavidin. In order to check for this possibility, two different protocols, using different tools, were carried out.

In one set of experiments (Fig. 3.11a), neurons co-expressing biosyn and sypHy were first stimulated with 40 APs at 20Hz, followed by the labelling of the entire recycling pool with strep594 in response to 900 APs at 20 Hz (Fig. 3.11b). After washing away the excess strep594, neurons were further stimulated with 40 APs at 20 Hz. As shown in figure 3.11c, the shape and the amplitude of sypHy responses to 40 APs at 20 Hz were very similar, before (green) and after (red) biosyn labelling.

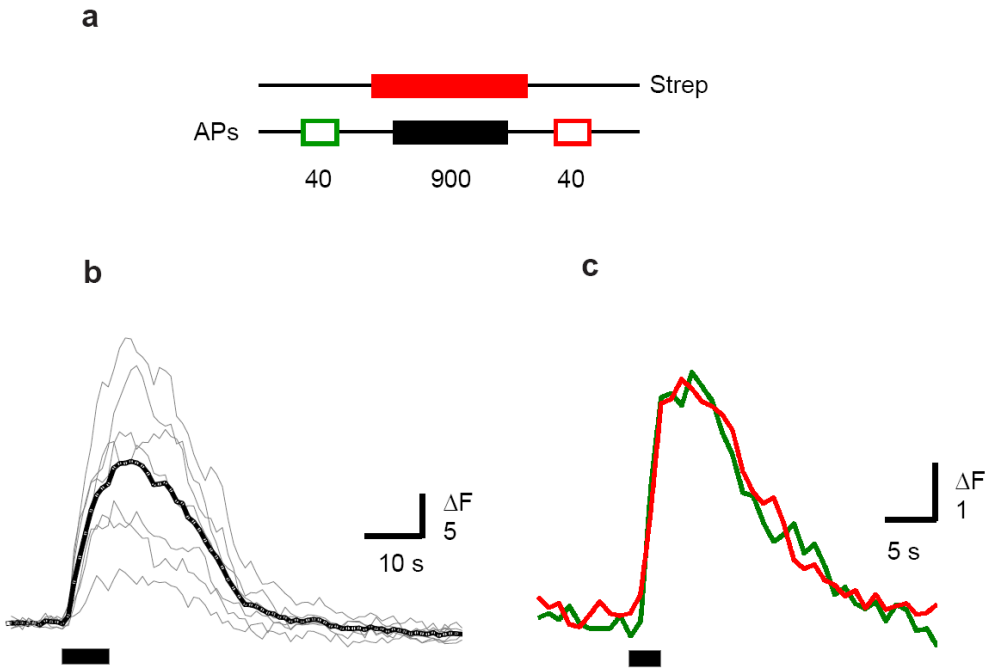


Figure 3.11: Biosyn labelling does not affect vesicle cycling. (**a**) Schematic diagram showing the time line of the experimental protocol: neurons were first stimulated with 40APs at 20Hz (open green box), followed by 900APs at 20 Hz (black box) in the presence of strep555 to label biosyn. After washing to remove excess streptavidin, neurons were once again stimulated with 40 APs at 20 Hz (open red box). (**b**) Responses to 900 APs at 20 Hz (black bar) measured from all synapses analysed from a single cell (gray) and the average response overlayed in black. (**c**) Overlay of the responses to 40 APs at 20 Hz (black bar) before (green) and after (red) labelling the entire recycling pool with streptavidin. Note the amplitude and rates of exocytosis and endocytosis are very similar.

In another set of experiments, neurons expressing biosyn were depolarized twice in the presence of strep488 (Fig. 3.12 a). This resulted in synapses strongly labelled in green (Fig. 3.12a). Strep488 was then washed away and neurons were left to rest for 5 minutes in order for the synapses to recover from the strong stimulations. The same synapses were then depolarized in the presence of the styryl dye FM4-64 that has been widely used to study vesicle cycling (Gaffield and Betz, 2006). These lipophilic dyes bind reversibly to the plasma membrane and become fluorescent when attached to the phospholipid bilayer. This property allows exo- and endocytosis to be monitored quantitatively. Figure 3.12a shows clearly that synapses labelled with strep488 were also stained with FM4-64. Furthermore, no significant difference was found in the average fluorescence intensity of FM4-64 puncta, for synapses labelled with strep488 and neighbouring, unlabelled synapses (Fig. 3.12b). Finally, a strong correlation was found between the fluorescence intensity of strep488 and FM4-64 at the same synapses (Fig. 3.12c), which provides further evidence that the same set of vesicles are being reused after labelling with strep488.

Taken together, these results provide strong evidence that synapses labelled with biosyn retain the ability to perform vesicle cycling normally.

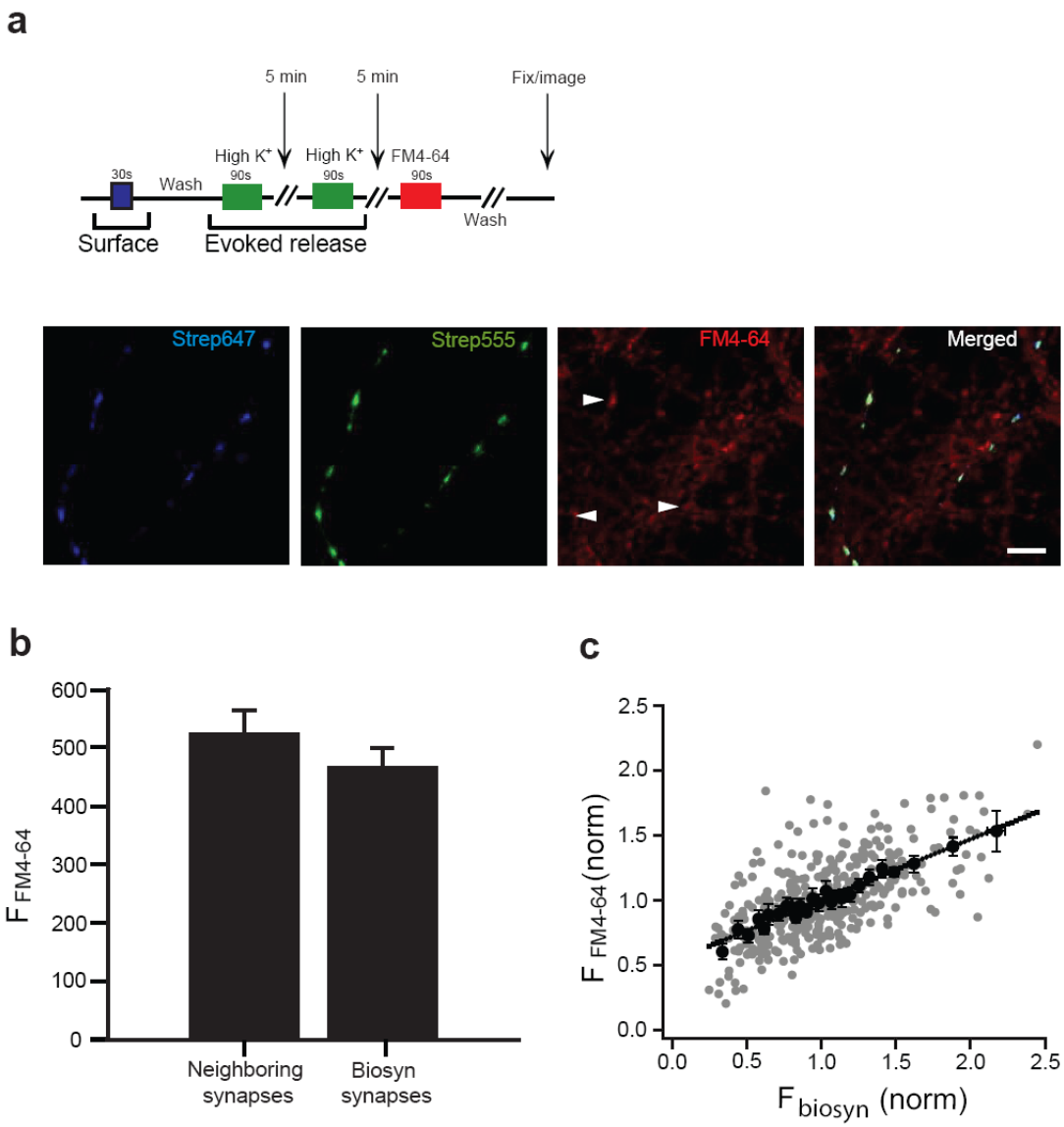


Figure 3.12: FM dyes show that biosyn labelling does not affect vesicle cycling. (**a**) Example images of presynaptic terminals where the surface labelling of biosyn is shown in blue and the labelling in response to two high K^+ depolarizations with strep488 is shown in green. The same synapses where subsequently depolarized with a single high K^+ depolarization in the presence of FM4-64, as shown in red. White arrows indicate example synapses clearly labelled with strep488 and also stained with FM4-64. Scale bar = 5µm (**b**) Average fluorescence intensity of FM4-64 puncta for synapses labelled with biosyn and neighbouring, unlabelled synapses, showing no significant difference between the two groups (unpaired T-test p = 0.2377). (**c**) Graph showing fluorescence intensity of FM4-64 staining as a function of biosyn labelling (gray dots are individual synapses; black circles are binned averages). The dotted line represents the best linear fit to the data.

3.6-Discussion

3.6.1- Summary of results

In this chapter, I have characterised the applicability of a new genetically-encoded probe, developed in the laboratory to study vesicle cycling, with single synapse resolution, in cultured hippocampal neurons. This reporter system is based on the *in vivo* biotinylation of VAMP2 (a probe termed 'biosyn') to irreversibly tag newly exocytosed vesicles with fluorescently labelled streptavidin. I have shown that the overexpression of the fusion protein VAMP2-BAP and its biotinylation do not interfere with the function of endogenous VAMP2 and neither does labelling with fluorescent streptavidin. More importantly, biosyn labelling was found to faithfully report the amount of exocytosis at the synapse.

3.6.2- Site-specific labelling of proteins in live cells

The labelling of proteins of interest at specific sites has provided researchers with a useful line of approach to visualise and study their dynamics with good spatial resolution in living neurons (Tsien, 1998; Chen and Ting, 2005). Our genetic approach is based on that principle and exploits the strong interaction that exists between biotin and streptavidin. In this case, our protein of interest, VAMP2, is expressed with the biotin acceptor peptide (BAP) fused to its luminal domain. The co-expression of this construct with the *E. coli* enzyme biotin ligase (BirA), which catalyzes the ligation of biotin to the acceptor peptide BAP, results in the luminal biotinylation of VAMP2, called biosyn. Fluorescently tagged streptavidin was used to irreversibly label biosyn expressed at the cell surface in a live preparation. A similar method has already been successfully applied to detect surface antibodies (Predonzani et al., 2008) and other transmembrane proteins such as post-synaptic receptors (Chen et al., 2005). In the latter study, recombinant BirA was not expressed within the cell, but instead was added to the medium to biotinylate post-synaptic receptors containing an extracellular BAP domain. This method requires a two-step labeling protocol. The first step comprises biotinylation of surface proteins by adding exogenous BirA, followed by labeling of the biotinylated protein with conjugated streptavidin. In the method used here, which is adapted from that used by Predonzani *et al.* (Predonzani et al., 2008), BirA was genetically-encoded and expressed directly in the cell, which resulted in a more convenient one-step (streptavidin labeling) protocol. Finally, this site-specific biotinylation approach to label proteins of interest has also been effectively used *in vitro* for protein detection by

western blots and for protein purification directly from animal tissues obtained from transgenic mice expressing cytosolic BirA (de Boer et al., 2003).

A number of other strategies have also been developed for *in vivo* labeling of proteins. As with biosyn, they are generally based on the use of diverse small tags attached to the proteins of interest, which will then act as ligands for exogenous labels. For example, Miller *et al.* have developed an approach that relies on the non-covalent interaction between dihydrofolate reductase (DHFR) and its ligand methotrexate (Miller et al., 2004). This method consists of fusing DHFR to a protein of interest which is then labeled using the cell-permeable methotrexate conjugated to Texas-red. They used this method to label either the nuclear or the plasma membrane proteins in DHFR-deficient Chinese hamster ovary (CHO) cells. There are two main disadvantages of this method. First, methotrexate remains attached to DHFR for a short time (about one hour), therefore making long term imaging impossible. Second, DHFR-deficient cell lines must be used to avoid non-specific labeling of the endogenous, ubiquitously expressed DHFR, which would otherwise result in strong background labeling.

In another approach with similar logic, Keppler *et al.* made use of the irreversible and specific interaction between the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) and its substrate O^6-benzylguanine (BG) (Keppler et al., 2003; Keppler et al., 2004). This method consists in fusing hAGT to a protein of interest, which is then labeled with a fluorescently tagged BG. Using this approach, they could label hAGT fusion proteins in different subcellular localizations such as the nucleus, the cytosol, the plasma membrane and the cytoskeleton (Keppler et al., 2004). As opposed to the DHFR/methotrexate interaction, hAGT covalently interacts with BG, therefore enabling a more prolonged imaging of labeled hAGT fusion proteins (for several hours). However, there are two main disadvantages with this approach. First, one of the main features of hAGT is its degradation after interacting with BG, which limits the time for visualizing hAGT fusion proteins after labeling. Second, similarly to DHFR, hAGT is based on an endogenous mammalian protein and can only be applied in hAGT-deficient cells to avoid BG reaction with endogenous AGT.

An additional approach that uses endogenously expressed domains to label proteins made use of extracellular tags, such as the highly specific nicotinic acetylcholine receptor (AChR) α-bungarotoxin (BTX) binding site. BTX, a snake venom toxin, binds

tightly to AChRs at a specific site on the channel. Recently, a 13 aa sequence homologous to the AChR-specific BTX binding site has been described (Harel et al., 2001). This 13 aa sequence has recently been fused to the extracellular domain of glutamate (AMPA) receptors and used to study AMPA receptor trafficking (Sekine-Aizawa and Huganir, 2004). The main disadvantage of this method is the possible cross-reactivity of BTX with endogenous nicotinic acetylcholine receptors, which would result in none specific labeling.

Finally, Tsien and colleagues have developed an alternative mechanism to label proteins of interest based on the covalent interaction between a small tetracysteine amino acid motif (only 6 aa long) and its ligand, the fluorogenic biarsenical (FIAsH) compounds (Griffin et al., 1998; Adams et al., 2002). FIAsH is membrane-permeable and becomes fluorescent upon binding to the tetracysteine motif. This approach consists of fusing the tetracyteine motif to a target protein, which is then labeled upon addition of the FIAsH dye (or its red-shifted analogue ReAsH). This technique has been used to study the trafficking of synaptotagmin 1 (Poskanzer et al., 2003) and AMPA receptors (Ju et al., 2004). The main advantage of this method is that it uses a small tag that is minimally-invasive and is unlikely to interfere with protein function. However, it has two major drawbacks that make its use limited: background staining due to endogenous cystein motifs and biarsenical toxicity.

In general, the common problem of all the techniques described above is that they use domains that are found endogenously and therefore give background staining. This poses specificity problems, which poses important limits on the interpretation of the results obtained.

In order to overcome the issue of non-specific binding, Marks *et al.* (Marks et al., 2004) have developed an alternative approach based on the highly specific interaction between a Phe36Val mutant of the immunophilin FK506 binding protein 12 (FKBP12(F36V)) and its synthetic ligand SLF', an analog of FK506 that does not bind to endogenous FKBP12 (Clackson et al., 1998). This approach consists in fusing FKBP12(F36V) to a protein of interest which is then labeled with a SLF' conjugated to a fluorescein. Marks *et al.* have successfully labeled FKBP12(F36V) fusion proteins in the nucleus, the cytosol and the plasma membrane in a large variety of cell lines including NIH 3T3, COS-7, C2C12, HeLa and Jurkat (Clackson et al., 1998). The main drawback of this

approach is that the ligand dissociates from the tagged protein which poses problems for long-term imaging.

The method we developed has a number of advantages over the above-mentioned techniques. It is characterized by a higher affinity between the tag and the fluorescent label ($K_d \sim 10^{-15}$ mol/L), which makes the interaction faster (in the milliseconds to hundreds of milliseconds range), more sensitive and more stable, enabling long-term imaging. Furthermore, the high specificity of the biotin-streptavidin interaction and the absence of endogenous biotinylated transmembrane proteins prevent the non-specific binding of the fluorescent label, resulting in an improved signal-to-noise ratio. Finally, our reporter system used a small tag that did not alter protein function. In summary, our new methodology is a highly efficient approach for the site-specific tagging of transmembrane proteins. In this chapter we showed its suitability for studying vesicle cycling in presynaptic terminals.

3.6.3- Studying vesicle cycling

In this chapter, biosyn was successfully used to label synaptic vesicles at single presynaptic terminal without affecting the release machinery. To date, three other techniques exist to study synaptic vesicle cycling with fluorescent imaging: Syt1-Abs, FM dyes and pHluorin-tagged synaptic vesicles proteins. However, these techniques differ in their ability to probe synaptic function.

Our approach is based on a genetically-encoded probe that specifically targets synaptic vesicles. For that reason, biosyn will label exocytic events that are due to synaptic release. Indeed, I showed that the vesicles that fused with the plasma membrane in response to a depolarizing stimulus were labelled with streptavidin whereas no vesicles were stained in the absence of activity. This result demonstrates the specificity of biosyn for studying synapse function. Among the other techniques available to study vesicle cycling, SypHy, which is also a genetically-encoded probe specifically targeted to synaptic vesicles (Granseth et al., 2006) and Syt1-Abs (Matteoli et al., 1992) also permit the specific labelling of synaptic vesicles. In contrast, FM dyes are less specific since they label all endocytic membrane events regardless of whether they are synaptic vesicles releasing neurotransmitter (Betz and Bewick, 1992). Nevertheless, they are useful probes for measuring relatively fast (seconds range) vesicle cycling events, where slow, non-synaptic endocytic events are not predominant. At the presynaptic terminal,

FM-dyes have been well characterised and shown to mainly report vesicle cycling events.

An important advantage of biosyn is that subsets of vesicles can be labeled irreversibly using streptavidin conjugated to different fluorophores, and that long term imaging can be performed to follow their axonal location over time (Howarth et al., 2008). Syt1-Abs also allow monitoring vesicle traffic over a long period of time in mature synapses and in developing axons (Kraszewski et al., 1995). However, the use of Syt1-Abs to label different subsets of synaptic vesicles is impractical due to the requirement for several antibodies directed against different epitopes of the same molecule. This poses the problem of non-specific labelling if the different epitopes are not sufficiently different to avoid cross-reactions between antibodies. A second issue is the steric hindrance that might impair the simultaneous interaction of all antibodies with synaptotagmin. Other techniques, such as SypHy and FM dyes do not allow for sequential labeling. Therefore, biosyn is the only reliable technique available to simultaneously label and study the different pools of vesicles at the synapse.

As shown in this chapter, biosyn does reliably label exocytosed vesicles in response to neuronal activity, but it cannot asses the dynamics of vesicle cycling in real time. Indeed, the labelling of vesicles with biosyn requires the removal of unbound fluorescent streptavidin to visualize exocytosis, thus restricting this technique to endpoint measurements. Until now, all the data obtained on vesicle recycling dynamics at individual synaptic terminals have relied on the use of FM-styryl dyes and pHluorins. These approaches have yielded important information regarding the kinetics of exo- and endocytosis at the synapse (Ryan et al., 1996; Klingauf et al., 1998; Murthy and Stevens, 1998; Sankaranarayanan and Ryan, 2000; Groemer and Klingauf, 2007; Dittman and Ryan, 2009). pHluorin-based probes in particular enabled real-time measurements of vesicle cycling dynamics, giving direct information on exocytosis and endocytosis, in response to a stimulation (Burrone et al., 2006; Balaji and Ryan, 2007; Groemer and Klingauf, 2007). FM dyes on the other hand can measure exocytosis but do not allow for direct measurements of endocytosis. However, they have been used to indirectly measure the time course of endocytosis in hippocampal neurons in culture (Ryan et al., 1996).

FM dyes and pHluorins have been widely used in dissociated hippocampal cultures to visualize vesicle cycling at a single release site (Ryan, 2001; Dreosti and Lagnado, 2011). This model has been instrumental in our understanding of vesicle recycling because it is easily amenable to high resolution imaging. I have shown that, similar to FM dyes and pHluorins, biosyn can be used to label and establish the relative size of vesicle pools that are released in response to particular stimulation protocols at single synapses in cultured neurons. Studying vesicle cycling in brain slices or *in vivo* would provide a better comprehension of synaptic function in physiological conditions. However, using biosyn in these models would be more challenging due to the need for extracellular application of fluorescently-tagged streptavidin, and to the restricted accessibility to the biotinylated transmembrane protein deep in tissue. Likewise, the use of FM dyes in more intact preparations has proven to be difficult due to the intrinsic lipophilic property of these dyes that result in a non-specific fluorescent staining of membrane surfaces. This renders the analysis of individual synapses more complex despite the development of extracellular quenchers of FM fluorescence (Pyle et al., 1999). By contrast, SpH has been successfully used to image synaptic activity *in vivo* in the olfactory bulb of transgenic mice expressing spH in olfactory receptor neurons (Bozza et al., 2004).

The biosyn technique could serve as a base for further studies of synaptic function. One possible application is its use in combination with electron microscopy. Ultrastructural analysis at a single vesicle resolution with electron microscopy has permitted considerable progress in our understanding of exocytosis and endocytosis at the synaptic terminal. The study of vesicle cycling has been made possible by the photoconversion of FM1-43 into an electron-dense product, thus allowing the visualization of recycling vesicles under the electron microscope (Harata et al., 2001). The main drawback of this approach is the high background staining. Instead, biosyn could be used to label vesicles with streptavidin simultaneously tagged with differently-sized gold particles and fluorescent dyes. This would allow the establishment of the spatial localization of recycling vesicles within the synapse by correlative light and electron microscopy (Darcy et al., 2006).

Finally, this biotinylation technology could also be easily used to tag any other transmembrane protein present at the presynaptic terminal and involved in neurotransmitter release, such as synaptotagmin, vglut and synaptophysin. This would

provide new insight into the role of these proteins in the presynaptic function. For instance, it would be interesting to compare the distribution and dynamics of these different proteins in experimental conditions similar to the ones we used in this study. As any other genetically-encoded probe, biosyn has the advantage of having a high spatial selectivity. Indeed, one could imagine targeting the enzyme birA to the surface of the pre- or the post- synaptic terminal and label a BAP-fused membrane protein on the opposite side. This could be useful for looking at synapse formation and might allow us to pinpoint the time at which the presynaptic side meets the post synaptic side.

In conclusion, I have shown that biosyn can be used to study vesicle recycling with comparative advantages over existing techniques such as FM dyes and pHluorins. The next chapter will exploit this new tool to investigate presynaptic function in hippocampal neurons.

CHAPTER IV

Independent vesicle pools underlie spontaneous and evoked release

4.1- Introduction

The presynaptic terminal contains about 200 synaptic vesicles that are thought to be organised into different pools (Sudhof, 2000): the readily releasable pool (RRP), the reserve pool (RP) and the resting pool. The RRP and the RP constitute what is known as the recycling pool, the vesicles that recycle in response to neuronal activity. The RRP are thought to correspond to the docked vesicles at the presynaptic active zone that are primed for release (Schikorski and Stevens, 1997; Rizzoli and Betz, 2005) and are therefore immediately available in response to stimulation, such as the arrival of an action potential (Rosenmund and Stevens, 1996). The RP is recruited in response to more sustained stimulation and serves to refill the RRP. Photoconversion of vesicles stained with the styryl dye FM1-43, together with electron microscopy, have established that the entire recycling pool represents only a subset (20 to 60%) of all the vesicles found at a presynaptic bouton in cultured hippocampal neurons. The remaining vesicles cannot be mobilised in response to neuronal activity and constitute what has generally been referred to as the resting pool of vesicles (Harata et al., 2001). The function of this pool remains largely unknown (Sudhof, 2000).

Three modes of neurotransmitter release occur at the synapse: synchronous, asynchronous and spontaneous release. Synchronous release is triggered by the arrival of an action potential, resulting in an increase in intracellular Ca^{2+} at the presynaptic terminal. This results in the synchronous fusion of vesicles with the plasma membrane and the release of neurotransmitter into the synaptic cleft. The tight temporal coupling between Ca^{2+} influx and vesicle fusion results in neurotransmitter being released with sub-milliseconds delays, ensuring fast communication between neurons (Sabatini and Regehr, 1999). Asynchronous release, also known as “delayed release”, follows synchronous release. It is driven by the residual, more global levels, of Ca^{2+} that occur in presynaptic terminals following the action potential. This form of release lasts for tens of milliseconds to seconds and is also Ca^{2+}-dependent, although to much lower

levels of intracellular Ca^{2+} (Del Castillo and Katz, 1954; Goda and Stevens, 1994; Atluri and Regehr, 1998; Lu and Trussell, 2000). Finally, a constitutive form of spontaneous release occurs in synapses at rest, in the absence of action potentials. It is best described by the fusion of single vesicles with the plasma membrane that results in miniature postsynaptic potentials (or 'minis') that reflect the release of a single quanta of neurotransmitter. This form of release happens with low probability and is thought to originate from the same pool of vesicles that are used for evoked release (Del Castillo and Katz, 1954).

However, recent findings have challenged the notion that the same pools of vesicles are responsible for all forms of release at the synapse. Kavalali and colleagues, using a combination of FM dyes and electrophysiology, showed that evoked and spontaneous release depend on two independent pools of vesicles with different release kinetics (Sara et al., 2005). In this study, vesicles loaded spontaneously with FM2-10 dye, destained much more slowly after stimulation than those vesicles loaded by means of activity. The authors concluded that spontaneously loaded vesicles belong to a specific pool not mobilised by action potentials. These findings were subsequently challenged by Klingauf and colleagues (Groemer and Klingauf, 2007). Following the loading of two spectrally distinct FM dyes, one loaded spontaneously and the other in response to activity at the same bouton, they found that the destaining rate profiles for both labelling conditions were identical, suggesting that both fusion events originate from the same pool of vesicles (Groemer and Klingauf, 2007). The origins of vesicles contributing to evoked and spontaneous release still remains highly controversial. A significant contributing factor to this controversy has been the limitations of the techniques available. However, the concept of whether or not there are different pools of vesicles supporting different modes of neurotransmitter release will have major consequences for our understanding of synapse function, including synaptic plasticity and development. In addition, it may also offer insights into the significance of miniature postsynaptic potentials in neurons.

The following questions will be addressed in this chapter: (1) do evoked and spontaneous release use two different vesicle pools? and (2) if so, what is the identity of these pools? For this purpose, the new biosyn technique described above (see chapter III) will be used in combination with the existing optical tools (pHluorin-based probes and FM dyes).

4.2- Two modes of release at the presynaptic terminal

As demonstrated in chapter III, biosyn is a reliable tool for labelling exocytosed vesicles in response to neuronal activity at a single presynaptic terminal without affecting the release machinery. However, it was also important to establish whether spontaneous fusion events could be reliably detected using biosyn too. This was particularly important since many of the available tools have been shown to have many drawbacks, such as lack of sensitivity or problems with background staining when using long exposure to dyes to measure the infrequent events that constitute spontaneous release.

4.2.1- Biosyn labels vesicles that fuse spontaneously

At 14 DIV, mature dissociated hippocampal neurons expressing sypHy and biosyn were first incubated in extracellular solution containing strep647 (in HBS) for 30s to quench surface biosyn. After washing out the excess of strep647, neurons were incubated for 15 min at 37 °C in HBS containing strep555, APV, CNQX, TTX and in the absence of Ca^{2+}, to avoid any activity-dependent fusion events (Fig. 4.1a). Figure 4.1b clearly shows that following this treatment, spontaneous uptake of strep555 (red) co-localises with the presynaptic marker sypHy (Fig. 4.1b). This result demonstrates that biosyn is sensitive enough to detect spontaneous fusion events that happen at individual synapses.

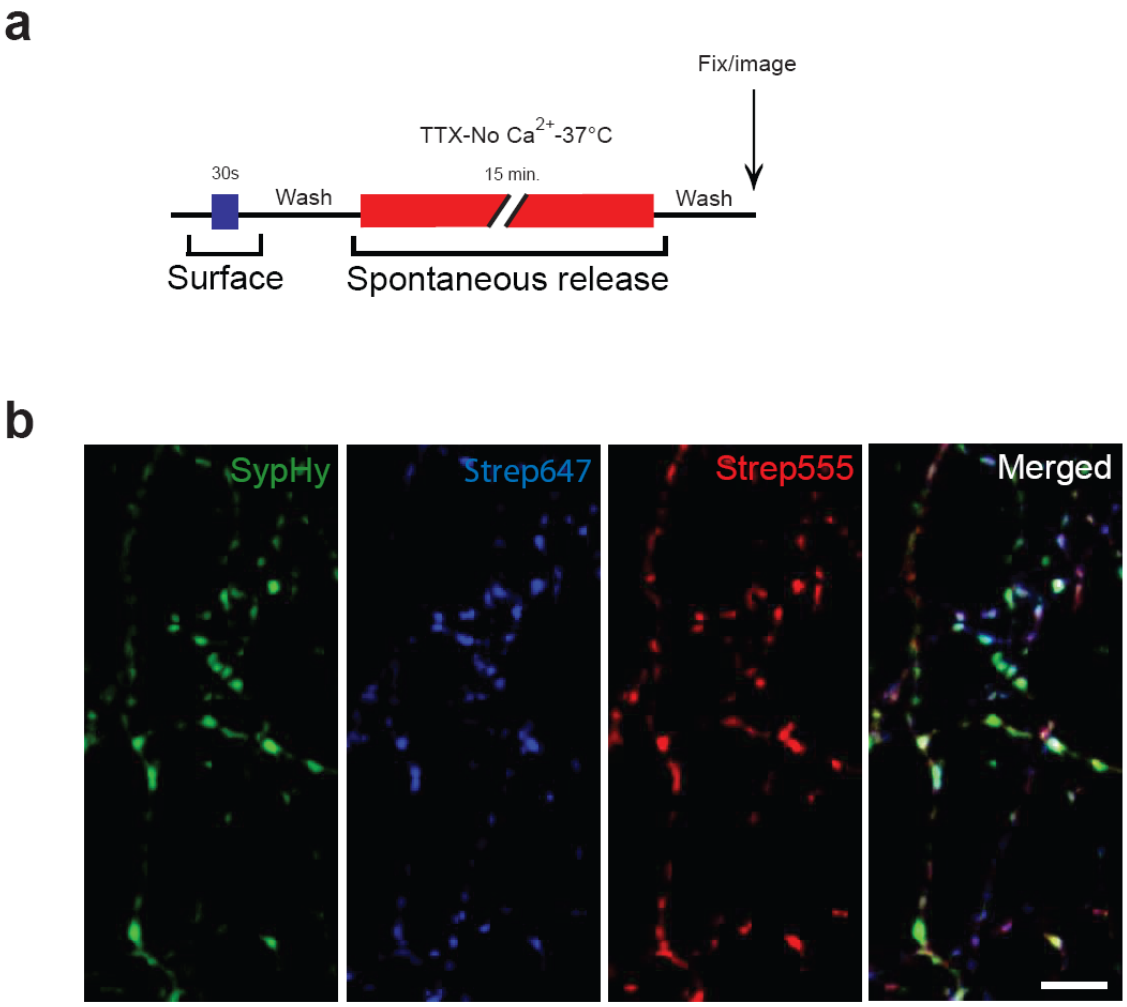


Figure 4.1: Labelling vesicles released spontaneously with biosyn. (**a**) Schematic diagram showing the time-line of the experimental protocol. Surface biosyn was stained with strep647 (blue) for 30s and washed. Neurons were then incubated for 15 min at 37°C in the presence of strep555, TTX and in the absence of Ca^{2+} to label spontaneous vesicle fusion. After washing, neurons were fixed and imaged. (**b**) Hippocampal neurons were co-transfected with biosyn and sypHy. Images show individual synapses expressing sypHy (green), spontaneous vesicle fusion events labelled with strep555 (red) and surface biosyn labelled with strep647 (blue). Scale bar = 5 µm.

4.2.2- Characterising spontaneous vesicle fusion events

Having established that biosyn was sufficiently sensitive to label spontaneous release, this probe was then used to measure a time-course for spontaneous vesicle recycling and further characterise these fusion events. After saturating surface biosyn with strep488, DIV 14 hippocampal neurons were treated for different periods of time with strep555 at 37°C, in the same conditions as above. The graph in figure 4.2a shows that the average fluorescence intensity of individual puncta labelled with strep555 increases with time until it reaches a plateau. The data could be fitted with a single exponential function with a time-constant of 6.2 minutes at 37°C. These results suggest that there is a finite pool of vesicles released spontaneously. However, after 15 min at room temperature (≈ 23°C) only 55% of the spontaneous pool was labelled, suggesting a much slower time constant.

To further confirm these findings, additional experiments were performed using the genetically-encoded probe sypHy. The alkaline trap method was used to establish the gradual fusion of vesicles with the plasma membrane during a 15 min exposure to bafilomycin. The gradual increase in fluorescence should represent the summed exocytic events at the synapse. Time-lapse imaging of sypHy positive presynaptic terminals was performed at 37°C for 20 minutes. The addition of bafilomycin after 5 min of baseline imaging (Fig. 4.2b, black bar below the graph) resulted in an exponential increase in sypHy fluorescence with a time constant of 4.8 min (Fig. 4.2b). The gray traces represent the change of sypHy fluorescence for each synapse normalized to the maximum value and the black trace is the average of all these synapses. Therefore, similar results were obtained using two independent technologies: biosyn and sypHy, indicating that a finite pool of vesicles is released spontaneously in about 6 minutes and certainly within 10 minutes. This finding is in accordance with previously published work (Sara et al., 2005; Groemer and Klingauf, 2007).

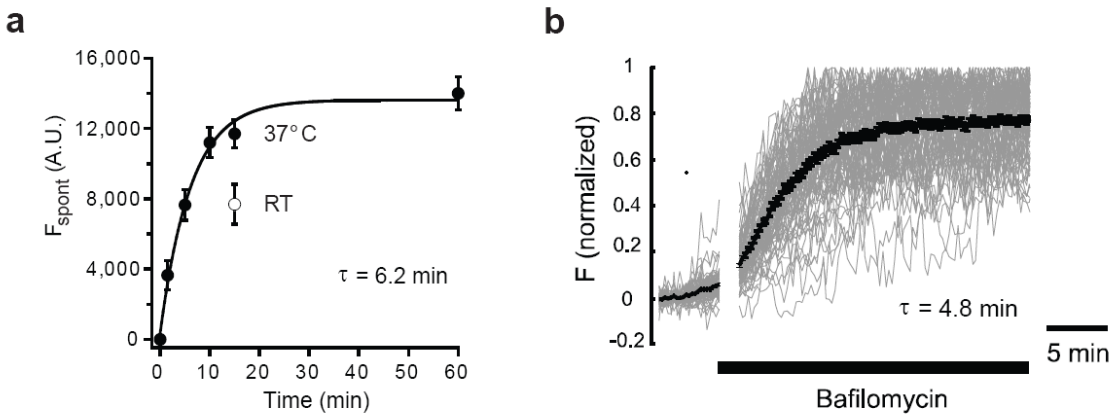


Figure 4.2: Time course of spontaneous vesicle fusion. (**a**) Hippocampal neurons transfected with biosyn were treated with strep555 for different periods of time. The graph shows the fluorescence intensity of spontaneous labelling as a function of time exposed to strep555. The data was fit with a single exponential function from which the time constant was established (τ = 6.2 min at 37°C). The filled circles represent n= 18-25 cells per condition treated at 37°C. The open circle corresponds to a single point at 15 min carried out at RT (≈ 23 °C) (n= 17 cells). Values are shown as mean ± s.e.m. (**b**) Hippocampal neurons were transfected with sypHy. The graph shows the spontaneous increase in sypHy fluorescence after addition of bafilomycin (black bar) measured for individual synapses (gray traces) with the average change in fluorescence (black trace) overlaid. The fluorescence change for each synapse (gray traces) is normalized to the maximum value for each trace. The curve was fit with a single exponential function from which the time constant was established (τ = 4.8 min at 37°C).

The next step was to investigate whether long-term treatment of neurons with bafilomycin could interfere with the release machinery and therefore modify the kinetics of spontaneous exocytosis. DIV 14 hippocampal neurons expressing sypHy and biosyn first had their surface biosyn labelled with strep647 for 30s. After washing out the excess of strep647, neurons were left for 3 min at 37°C in HBS without Ca^{2+}, in the presence of TTX and strep555 to label spontaneous vesicle fusion. This was done in the presence or absence of bafilomycin (Fig. 4.3a). In this experiment sypHy was used as a marker for presynaptic terminals. During the 3 min incubation spontaneous neurotransmitter release occurs without having reached saturation therefore allowing a direct comparison of strep555 uptake in both conditions. No difference was found in the average fluorescence intensity of spontaneous strep555 uptake between neurons treated with bafilomycin for 3 min and controls (P = 0.93) (Fig. 4.3b, c). This result indicates that the release machinery is not perturbed when cultures are treated for a long period of time with bafilomycin.

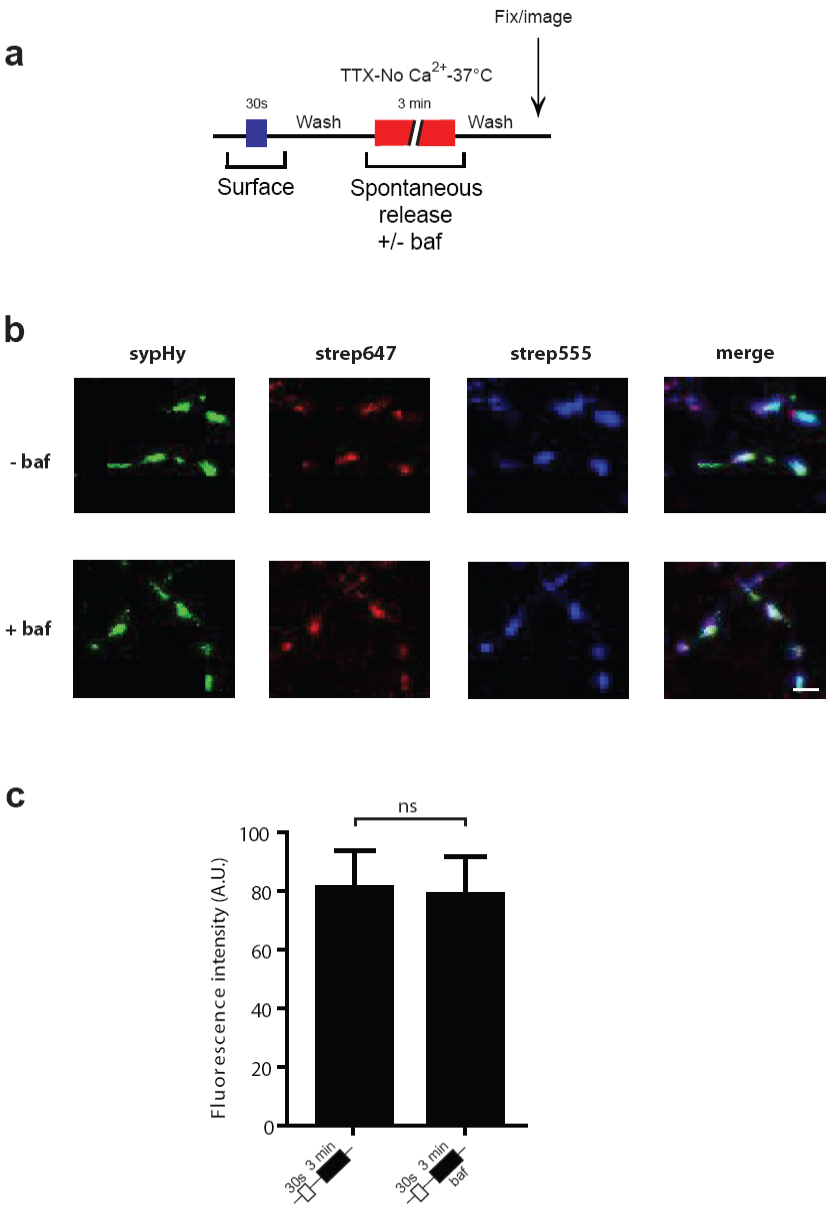


Figure 4.3: Bafilomycin has no effect on vesicle fusion. (**a**) Schematic diagram showing the time-line of the experimental protocol. After labelling surface biosyn with strep647 (blue) for 30s, spontaneous vesicle fusion was labelled with strep555 (red) in the presence of TTX, in the absence of Ca^{2+}, and incubated at 37°C for 3 min in the absence or presence of bafilomycin (baf). (**b**) Hippocampal neurons were co-transfected with biosyn and sypHy. Images show individual synapses expressing sypHy (green), spontaneous vesicle fusion events labelled with strep555 (red) after 3 min in the absence or presence of bafilomycin and surface biosyn labelled with strep647 (blue). Scale bar = 5 µm. (**c**) The graph shows the fluorescence intensity of synapses labelled with strep555 during 3 min of spontaneous release. The fluorescence intensity plotted corresponds to the treatment denoted as black squares. The first bar (from the left) shows the intensity of vesicles labelled spontaneously during 3 min in the absence of bafilomycin (n= 210 synapses from 6 cells). The second bar shows the intensity of vesicles labelled spontaneously during 3 min in the presence of bafilomycin (n= 155 synapses from 6 cells). Statistical analyses was performed using a paired t test; P = 0.93 (ns: non-significant). Values are shown as mean ± s.e.m.

The use of biosyn allowed a comparison between the size of evoked and spontaneous forms of release at individual synapses. This was done by measuring the mean fluorescence intensity for activity-dependent release in one set of neurons and the spontaneous release in another set of neurons in three independent experiments (ie: in neurons obtained from three different rats). The data was normalized to evoked release for each set of experiments as different fluorophores were used. As shown in figure 4.4, the size of the spontaneous pool of vesicles (0.46 ± 0.05; n = 21 neurons) was found to be approximately half the size of the evoked pool (1± 0.05; n = 64 neurons).

In summary, the results so far suggest that there is a finite pool of vesicles that is released spontaneously, that release from this pool saturates within 10 minutes and that it is approximately half the size of the evoked pool of vesicles.

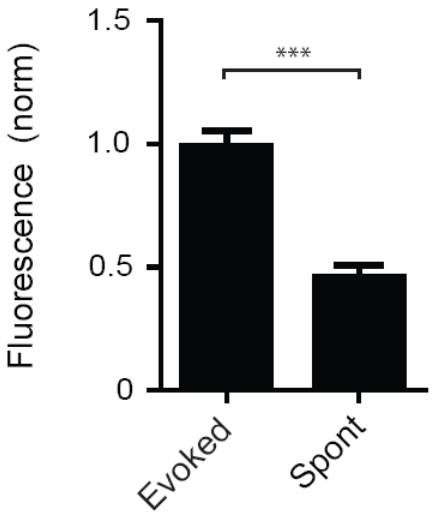


Figure 4.4: Evoked versus Spontaneous pool size. The graph shows the mean fluorescence intensity for activity-dependent release and spontaneous release (15 min time point at 37°C) (n > 400 synapses from 20 cells, for each condition). Statistical analyses were performed using Mann-Whitney test (*** P< 0.0001). Values are shown as mean ± s.e.m.

4.3- Two distinct pools of vesicles: spontaneous and evoked

If spontaneous and evoked forms of release were to originate from the same set of vesicles then one would expect the size of both pools to be equivalent. The fact that the spontaneous pool is finite and represents half the size of the evoked pool can be explained in one of two ways: either only a subset of vesicles that are released in response to neuronal activity are also capable of spontaneous release or each mode of release originates from two distinct pools of vesicles. Biosyn is ideally suited to distinguish between the two possibilities as it allows irreversible and sequential labelling of vesicles with different fluorescent probes on the same set of neurons. Thus, by saturating all of the biosyn binding sites for vesicles involved in one form of release, it is possible to define whether the number of vesicles implicated in the other form of release is changed.

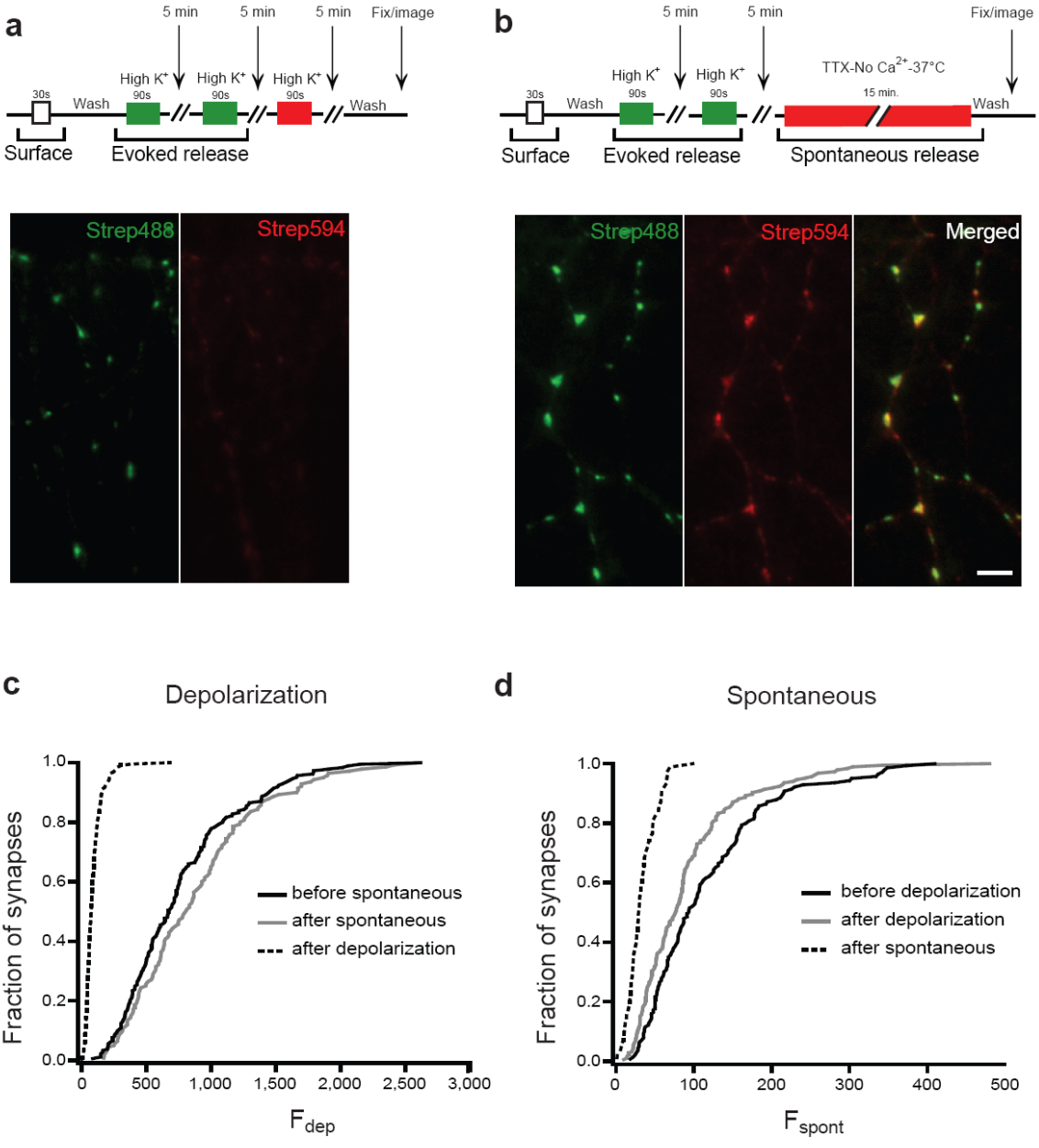
It was first necessary to check whether the stimulation protocol used to label vesicle pools was indeed saturating. DIV 14 hippocampal neurons expressing biosyn were used and the surface biosyn was quenched with unlabelled streptavidin for 30s. After unlabelled streptavidin was washed out, neurons were stimulated twice for 90s with a high potassium solution containing strep488, APV, CNQX and TTX (Fig. 4.5a). This resulted in a strong green fluorescent label. After washing away strep488, the neurons were further stimulated for 90s with a high potassium solution, this time containing strep594. As expected for a saturating stimulus, no further labelling with strep594 was observed, as all of the biosyn binding sites were already occupied by strep488 (Fig. 4.5a and dotted line in Fig. 4.5c). This result demonstrated that two consecutive depolarisations resulted in a saturating stimulus.

To investigate whether vesicles fusing spontaneously belong to the same set of vesicles as those recruited in response to a depolarising stimulus, the entire recycling pool was labelled with strep488 in a sister culture, as described above. After washing away the excess of strep488, neurons were incubated for 15 min at 37°C in HBS, without Ca^{2+}, containing APV, CNQX, TTX and strep594 to label vesicles that fuse spontaneously (Fig. 4.5b). There are two possible outcomes that can arise from this experiment: if both forms of release draw from the same set of vesicles then the presynaptic terminal should be labelled only in green as all the biosyn binding sites would be occupied by strep488. But, if they were to originate from two different pools of vesicles, then presynaptic

terminals would be labelled in both green (strep488) and red (strep594). As shown in the images in Fig. 4.5b, the synapses were indeed labelled in green and red, strongly suggesting that each form of release draws, at least in part, from two distinct pools.

To properly establish whether evoked and spontaneous form of release originate from two separate pools, different combinations of depolarisation and spontaneous treatment were applied to the neurons. If the two pools were truly independent of each other then the order in which they were labelled should not affect the amount of staining observed. As shown in figure 4.5d, there was no significant difference between the cumulative distribution of fluorescence intensity for spontaneous labelling, whether spontaneous vesicle fusion was measured before or after the recycling pool was released ($P > 0.05$) (Fig. 4.5d), suggesting that the recruitment of the evoked pool does not affect the size of the spontaneous pool. Similarly, the cumulative distribution of fluorescence intensity for the evoked pool was unaffected by the labelling of the spontaneous pool ($P > 0.05$) (Fig. 4.5c), but was decreased after the recycling pool was depleted ($P < 0.001$), once again confirming that the stimulation used is indeed saturating. Moreover, spontaneous labelling was clearly reduced when it was assessed after the spontaneous pool of vesicles was recruited ($P < 0.001$) demonstrating that the 15 minutes incubation to label the spontaneous pool is close to saturation (dotted line in Fig. 4.5d).

Figure 4.5: Two distinct pools of vesicles with different release modes: spontaneous and evoked. (**a**) Two consecutive depolarizations with strep488 (green) labelled the entire recycling pool. A further depolarization with strep594 (red) resulted in no further staining. (**b**) Labeling the recycling pool (green), followed by spontaneous labeling (red) resulted in a strong signal in both channels. Scale bar = 5 µm. (**c**) Example from one set of sister cultures showing the cumulative distribution of fluorescence intensity for synapses labeled with strep594 for spontaneous fusion before (solid black line, n = 140 synapses) and after (gray line, n = 185 synapses) depolarization (the median intensity was not significant between the two conditions, one-way ANOVA, $P > 0.05$). The dashed black line shows spontaneous labeling after the entire pool of spontaneous vesicles was labeled with another color (n = 85 synapses, statistically significant when compared with either of the two conditions plotted in the graph, one-way ANOVA, $P < 0.001$). (**d**) Cumulative distribution of fluorescence intensity for synapses from the same sister cultures labeled with strep488 using a depolarizing stimulus before (solid black line, n = 185 synapses) and after (gray line, n = 140 synapses) the spontaneous pool was released (the median intensity was not significant between the two conditions, one-way ANOVA, $P > 0.05$). The dashed line shows evoked labeling after the entire pool of evoked vesicles were labeled with another color (n = 263 synapses, statistically significant when compared with either of the two conditions plotted in the graph, one-way ANOVA, $P < 0.001$). Note that **c** and **d** use different fluorescent probes (strep594 and strep488, respectively). Fluorescence intensities can only be compared between curves of the same graph. Values are shown as mean ± s.e.m.


a
5 min
5 min
5 min
Fix/image
High K+
High K+
High K+
30s
90s
90s
90s
Wash
Wash
Surface
Evoked release
b
5 min
5 min
Fix/image
TTX-No Ca2+-37°C
High K+
High K+
15 min.
30s
90s
90s
Wash
Wash
Surface
Evoked release
Spontaneous release
Strep488
Strep594
Strep488
Strep594
Merged
c
Depolarization
Fraction of synapses
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1,000
1,500
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2,500
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Fdep
before spontaneous
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d
Spontaneous
Fraction of synapses
1.0
0.8
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100
200
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400
500
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before depolarization
after depolarization
after spontaneous

To reinforce the conclusion that both spontaneous and evoked form of release originate from two different pools, it was important to verify whether there were any vesicles that became competent for evoked release during the 15 min incubation used to label the spontaneous pool. Indeed, including the time taken to wash out the streptavidin, between the end of high K+ stimulation and the end of spontaneous labelling there was a gap of 18.5 min. In order to check for a possible recovery of the evoked pool during that time of incubation, two key experiments were performed.

In a first set of experiments the degree of saturation of a high k^+ stimulus after an 18.5 min delay was measured. DIV 14 hippocampal neurons expressing biosyn had their surface biosyn quenched by applying strep488 for 30s. After washing away the excess of strep488, neurons were depolarized twice in the presence of strep647 to label the entire recycling pool of vesicles (condition A in Fig. 4.6). This represents the control condition. In another set of neurons, after washing out excess strep647, neurons were incubated for 15 min at 37°C to allow spontaneous recycling in the absence of any streptavidin, after which neurons were depolarized in the presence of strep555 to label any evoked vesicles. The amount of strep555 fluorescence obtained after high K+ stimulation is depicted in graph 4.6 as condition C (Fig. 4.6C). In order to check whether this amount of release was due to activity alone, a second set of experiments was performed to establish the amount of surface biosyn present at the same time point. Neurons were processed in the same way, but this time, after spontaneous recycling, surface biosyn was labelled with strep555 for 30s. A small amount of surface biosyn was found on the plasma membrane and represents the biosyn left behind by vesicles that have fused spontaneously with the plasma membrane during the 18.5 min incubation period (condition B in Fig. 4.6). This surface biosyn represents an offset that must be subtracted from the amount of biosyn labelling obtained after depolarization, to give the true amount of activity dependent vesicle fusion. The final bar in graph 4.6 shows the amount of release due to activity alone (labelling due to depolarisation minus labelling due to surface staining). These results clearly show that the evoked pool of vesicles does not recover during the 15 min of spontaneous release and further establish that mixing of vesicles between pools does not occur within this time period. Finally, it is important to note that experiments were carried out to avoid as much as possible any mixing between the two pools by performing the depolarising stimuli at room temperature to minimise spontaneous fusion events, which are notoriously temperature sensitive (see Fig. 4.2a for temperature dependence of spontaneous release).

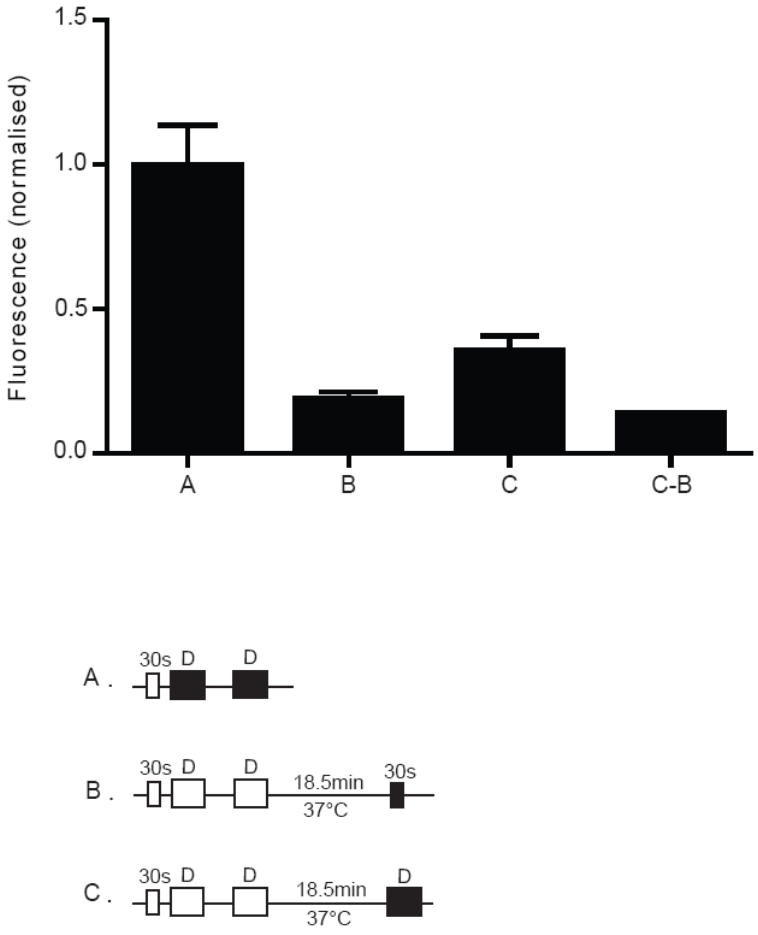


Figure 4.6: The recycling pool and the spontaneous pool do not mix within the time-course of the experiment. The graph shows the fluorescence intensity (normalized to the recycling pool) that results from the staining protocols shown in the diagrams below (A to C). (A) represents the fluorescence of the recycling pool. (B) is the fluorescence that is left on the surface of the plasma membrane after 18.5 min (15 min at 37°C plus washes) of spontaneous recycling, having previously labeled the entire recycling pool as in A. At the end of the 18.5 min period the surface biosyn is labeled (30s). The amount of staining observed represents the biosyn left behind on the surface of the plasma membrane during spontaneous vesicle cycling. C. represents the amount of evoked released that can be elicited at the same time point as B. The amount of staining observed represents the sum of the surface biosyn plus the amount released through neuronal activity. Subtracting B from C gives the amount of release elicited by neuronal activity only, which is shown as the final bar in the graph (C-B). Values are shown as mean ± s.e.m.

Using these saturating conditions it was possible to define the relative size and extent of mixing of each pool of vesicles in a mature synapse. DIV 14 hippocampal neurons expressing biosyn had their surface biosyn quenched by applying unlabelled streptavidin for 30s (Fig. 4.7 last bar on the right of the graph). After washing away the unlabelled streptavidin, neurons were treated using different protocols to label synapses with a depolarising stimulus (D) or during spontaneous release (S) (Fig. 4.7). The graph in figure 4.7 represents the quantification of the fluorescence intensity at each synapse for the different conditions (denoted as black squares below each bar), pooled from three independent experiments. Each condition was normalised to the size of the recycling pool for the different fluorophores used. The fluorescence intensity of spontaneous release was found to remain constant whether the spontaneous labelling was done alone (n = 21 cells), before (n = 27 cells) or after (n = 32 cells, $P > 0.05$) the recycling pool was recruited. This is consistent with the idea that spontaneous release uses a different set of vesicles from evoked release. Likewise, the fluorescence intensity of evoked release remains unaffected ($P > 0.05$) whether the labelling was done alone (n = 64 cells), before (n = 28 cells) or after (n = 32 cells) spontaneous labelling. Together, these results demonstrate that spontaneous vesicles are recruited from a different pool than that used by evoked release. Furthermore, the fluorescence intensity of the spontaneous labelling was significantly reduced when measured after the spontaneous pool was mobilised ($P < 0.001$). Similarly, the fluorescence intensity of evoked release was drastically decreased when assessed after the recycling pool was labelled ($P < 0.0001$), confirming that the recruitment of the recycling pool and the spontaneous pool was done using saturating conditions.

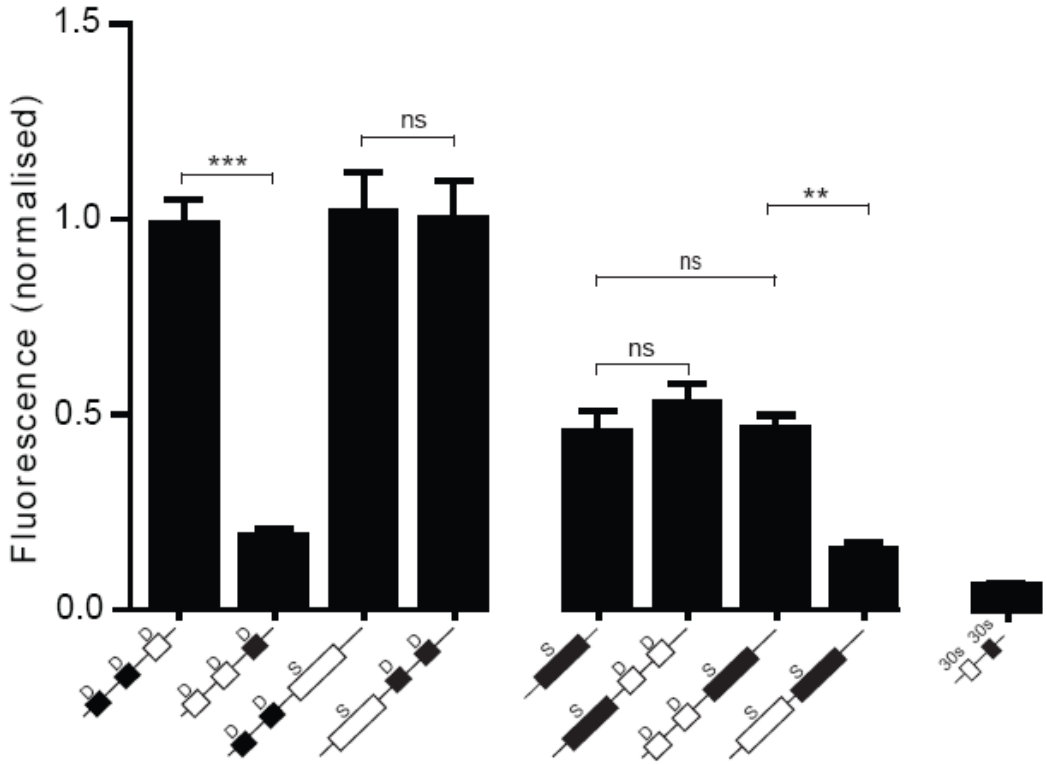


Figure 4.7: Quantification of fluorescence intensity for different conditions. The graph shows the fluorescence intensity of synapses labeled with a depolarizing stimulus (D) or during spontaneous release (S). Each condition involved several labeling protocols, the sequence of which is shown below each bar. The fluorescence intensity that is plotted corresponds to the treatments denoted as black squares, whereas the empty squares are treatments that are not plotted. The first two bars (from the left) show the intensity of vesicles labeled with depolarizing stimuli, delivered before or after another depolarizing stimulus (n = 64 neurons for each bar). The next two bars also represent the intensity of vesicles labeled with depolarizing stimuli, this time before or after spontaneous release (n = 28 and n = 32 neurons, respectively). The second set of four bars corresponds to the intensity of vesicles labeled spontaneously. Regardless of when spontaneous release was assessed (on its own, before or after a depolarizing stimulus) the total intensity of spontaneous labeling was very similar (n = 21, n = 27 and n = 32 neurons respectively). Note that spontaneous labeling that occurred after the spontaneous pool was mobilized was significantly reduced, indicating pool saturation. The final bar is a control to show that surface labeling of biosyn (30s exposure to streptavidin) saturated all biosyn binding sites (n = 14 neurons). Statistical analysis was performed using a nonparametric one-way ANOVA test (ns, not significant; $P > 0.05$). ** $P < 0.001$ and *** $P < 0.0001$. Values are shown as mean ± s.e.m.

4.4- Identity of the spontaneous pool

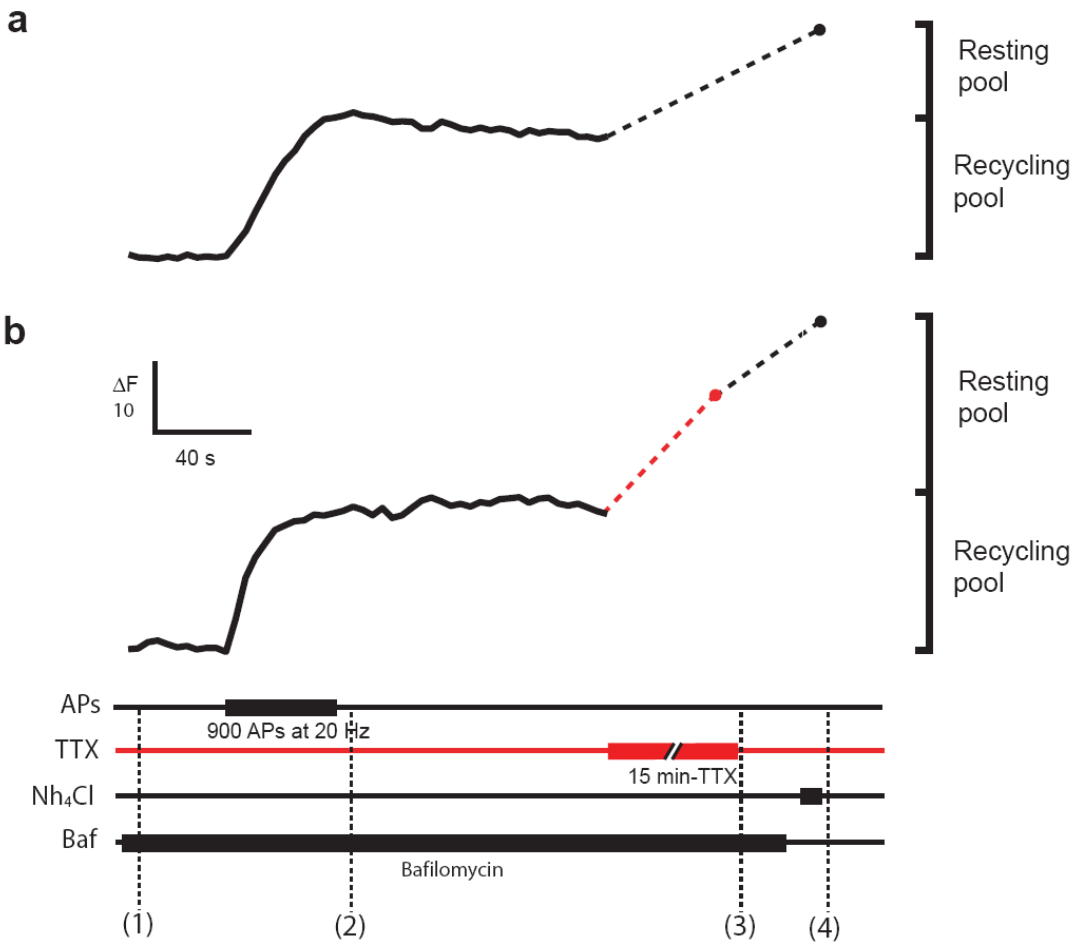
Synaptic vesicles can be mobilised either in response to neuronal activity, which draws from the recycling pool of vesicles, or spontaneously. Having established that they use different sets of vesicles, it was then important to establish the source of spontaneous vesicles. At hippocampal presynaptic terminals, the only other pool of vesicles that is not the recycling pool, is known as the resting pool (Sudhof, 2000), which cannot be mobilised in response to stimulation. These properties make it a strong candidate for the pool of vesicles used during spontaneous release.

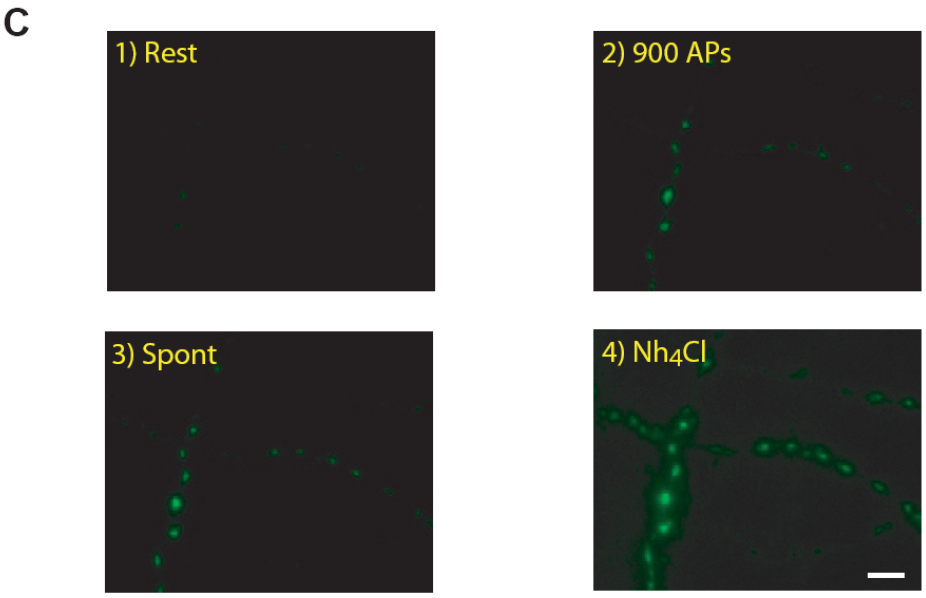
4.4.1- Defining the size of the resting pool

To identify the source of vesicles used during spontaneous release, it was first necessary to define the relative sizes of the recycling and resting pools of vesicles on DIV 14 hippocampal neurons. For this reason we employed a previously described method for measuring vesicle pool sizes that makes use of the reporter sypHy together with the alkaline trap method (Sankaranarayanan and Ryan, 2001). Previous uses of this approach employed the reporter synaptopHluorin to compare the size of vesicle pools in excitatory and inhibitory neurons, and provide a description of the modes of recycling used by the activity-dependent pool of vesicles (Li et al., 2005; Burrone et al., 2006). Here, we employed a similar technique, this time to establish the origin of the spontaneous pool.

Neurons expressing sypHy were stimulated in the presence of bafilomycin with a saturating stimulus of 900 action potentials at 20 Hz (see Fig. 3.8), which mobilizes the entire recycling pool, resulting in a measurable increase in fluorescence during the stimulus (Fig. 4.8a). The amplitude of the change in fluorescence corresponds to the size of the recycling pool of vesicles. In addition, any vesicles that were not released in response to the stimulation protocol could be unquenched by the application of ammonia (pH = 7.3) which equilibrates the pH across all membranes (Miesenbock et al., 1998). Indeed, the addition of NH_4Cl following the stimulation resulted in a further increase in fluorescence that represents the so-called ‘resting pool’ and which was about half the size of the recycling pool (Fig. 4.8a). This result is consistent with previous studies (Li et al., 2005).

Figure 4.8: Measuring vesicle pools with sypHy. (**a**) Changes in fluorescence from a single neuron in response to 900 action potentials at 20 Hz to mobilize the entire recycling pool. Unquenching the remaining vesicles with NH_4Cl uncovered the resting pool. (**b**) The same experimental protocol as in **a** was used, but this time allowed spontaneous release to occur after the recycling pool had been mobilized. A single neuron was stimulated with 900 action potentials at 20 Hz to mobilize the entire recycling pool. Synapses were then left for 15 min at 23°C in the presence of TTX to establish whether any vesicles could be released spontaneously. Finally, any remaining vesicles were unquenched by addition of NH_4Cl. The relative sizes of the recycling pool and the resting pool of vesicles are shown for each cell. (**c**) Example images of the presynaptic terminals from the neuron shown in **b** after certain treatments. Top left (1), synapses at rest. Top right (2), synapses after 900 action potentials at 20 Hz. Bottom left (3), synapses after spontaneous release for 15 min at 23°C in TTX and 2 mM Ca^{2+}. Bottom right (4), synapses after unquenching all vesicles with NH_4Cl. Scale bar = 5 µm.


a
Resting pool
Recycling pool
b
ΔF
10
40 s
Resting pool
Recycling pool
APs
900 APs at 20 Hz
TTX
15 min-TTX
Nh4Cl
Baf
Bafilomycin
(1)
(2)
(3)
(4)


C
1) Rest
2) 900 APs
3) Spont
4) Nh4Cl

4.4.2- Spontaneous fusion originates from the resting pool

A similar further experiment was performed to determine whether vesicles released spontaneously originate from the resting pool. Neurons were first stimulated in the presence of bafilomycin with 900 action potentials at 20 Hz to recruit the entire recycling pool of vesicles (Fig. 4.8b,c2). They were then left for a further 15 minutes at RT (≈ 23°) in the presence of TTX and bafilomycin to allow for spontaneous release to occur. This resulted in a further increase in fluorescence that can only originate from the resting pool as the entire recycling pool had already been mobilised in response to the saturating stimulus (Fig. 4.8b,c3). Since the experiment was carried out at room temperature (which is suboptimal for spontaneous release, see Fig. 4.2a), addition of ammonium chloride resulted in a further increase in fluorescence; representing the remaining vesicles in the resting pool that were not mobilized during the experiment (Fig. 4.8c4). The increase in fluorescence following spontaneous release represented 60% of the resting pool, consistent with the result obtained with biosyn where only 55% of the spontaneous pool of vesicles was labelled at RT (see Fig. 4.2a).

A summary of all experiments is presented in figure 4.9. It shows the average change of fluorescence for the different conditions, normalised to the size of the recycling pool. After the entire recycling pool was mobilised, in response to 900 action potentials at 20 Hz, neurons were either treated directly with ammonia (Fig. 4.9, filled circles, n = 6 cells) or allowed to release spontaneously for 15 minutes in the presence of TTX at 23°C and then treated with ammonia (Fig. 4.9, red trace, n = 7 cells). This graph clearly shows that on average, synapses have a resting pool that is approximately half the size of the recycling pool. In addition, the number of vesicles that were unquenched by the application of ammonia was reduced by two-thirds when spontaneous fusion was allowed to occur.

Together, these results, obtained using sypHy, confirm that there are two independent pools of vesicles that are released either spontaneously or in response to neuronal activity and that spontaneous release originates from the resting pool.

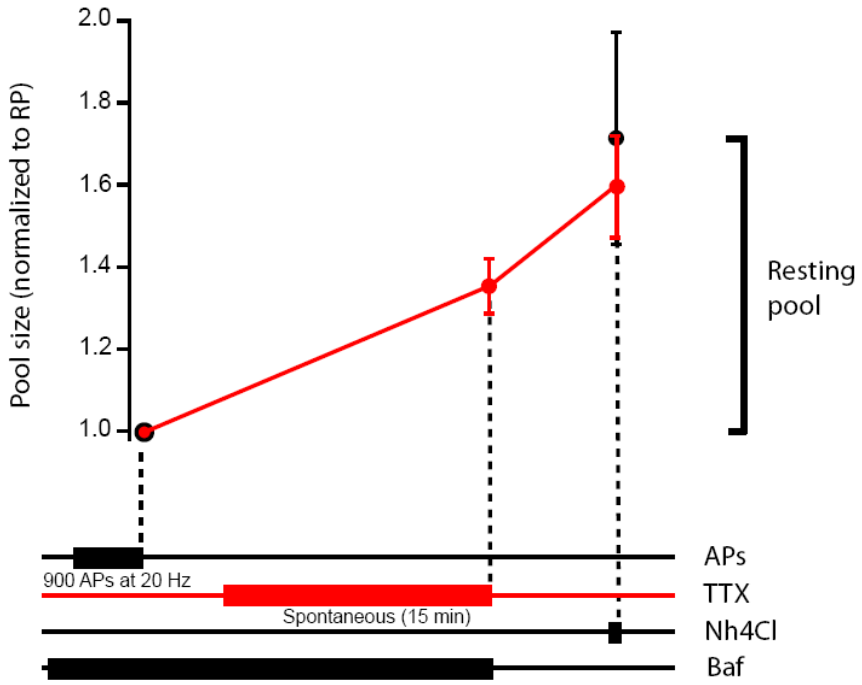


Figure 4.9: The spontaneous pool of vesicles corresponds to the resting pool. The graph shows a plot of the change in fluorescence (normalized to the recycling pool) following different treatments. After the entire recycling pool had been mobilized with a saturating stimulus of 900 action potentials at 20 Hz, neurons were either treated immediately with NH_4Cl (filled black circles, n = 6 cells) or allowed to release spontaneously for 15 min in TTX at 23°C and subsequently treated with NH_4Cl (red trace, n = 7 cells). The diagram below gives a temporal representation of the experimental protocol. Values are shown as mean ± s.e.m.

4.5 –Establishing the size of the spontaneous and evoked pools of vesicles

Having established that the evoked and spontaneous pool of vesicles is distinct, it was important next to understand how the size of each pool was controlled at each synapse. In other words, is there any correlation between the sizes of each pool or does each synapse regulate the size of the pools independently?

To tackle this question we performed experiments using the two reporters described above: biosyn and sypHy. In one set of experiments, DIV 14 hippocampal neurons expressing biosyn had their evoked and spontaneous pool of vesicles labelled sequentially using streptavidin conjugated to different fluorophores. The graph in Fig. 4.10a, represents a plot of the fluorescence intensity for spontaneous labelling as a function of the fluorescence intensity for evoked labelling, for individual synapses along an axon (n = 989 synapses from 26 cells). A significant correlation was found between the two variables (correlation coefficient = 0.57, $P < 0.05$).

In another set of experiments, DIV 14 hippocampal neurons expressing sypHy were first stimulated to release the entire recycling pool, followed by the recruitment of the spontaneous pool for 15 min at 23°C. Graph 4.10b shows a plot of the change in fluorescence amplitude for evoked release as a function of the change in fluorescence for spontaneous release at individual synapses (n = 71 synapses from 7 cells). A significant correlation was found between the total size of the evoked pool and the amount of spontaneous release that occurred during the 15 min period at 23°C (correlation coefficient = 0.62, $P < 0.05$).

Taken together, these results demonstrate, using two different approaches that the size of both pools of vesicles correlates meaning that the properties of spontaneous release may accurately reflect those of evoked release. This is in line with previous findings (Prange and Murphy, 1999; Sara et al., 2005), but does not agree with another study of Fernandez-Alfonso *et al.* results (Fernandez-Alfonso and Ryan, 2008). It is unclear what the reason for this discrepancy is.

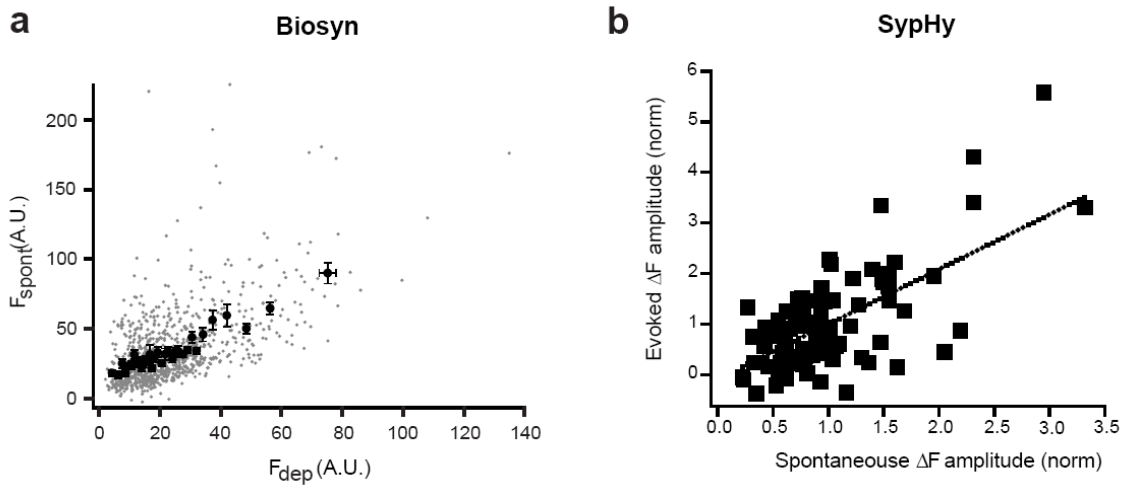


Figure 4.10: The size of spontaneous and evoked pool of vesicles correlates. (**a**) Hippocampal neurons were transfected with biosyn. Graph showing the fluorescence intensity of the spontaneous pool as a function of the fluorescence intensity of the recycling pool for individual synapses (gray dots) and synapses grouped in bins of 30 (black circles, n = 989 synapses from 26 cells). Correlation coefficient = 0.57, $P < 0.05$. (**b**) Hippocampal neurons were transfected with sypHy. Graph showing a plot of the change in fluorescence amplitude measured with sypHy for evoked release at individual synapses (black squares) as a function of the change in fluorescence for spontaneous release. The dotted black line represents the best linear fit to the data. Correlation coefficient = 0.62, $P < 0.05$. Values are shown as mean ± s.e.m.

4.6- VAMP2 requirement for evoked and spontaneous vesicle fusion

Previous studies have shown that VAMP-2 is needed for both evoked and spontaneous neurotransmitter release (Deitcher et al., 1998; Schoch et al., 2001). However, evoked release has generally been *more* sensitive to manipulations of VAMP2 activity than spontaneous release, showing a possible molecular difference between the two forms of release (Schoch et al., 2001). Work by Schiavo *et al.* clearly showed that Tetanus neurotoxin (TeNT) dramatically blocked evoked neurotransmitter release (Schiavo et al., 1992) and since then this toxin has been used extensively to perturb neurotransmitter release in neurons. The logic behind this experiment was to establish whether a synaptic vesicle requires VAMP-2 to spontaneously fuse with the plasma membrane. Further still, it also provided a means to control for possible non-specific increases in sypHy fluorescence when using the alkaline trap method. TeNT is formed of a heavy (Hc) and a light chain (Lc). The heavy chain binds specifically to the presynaptic terminal

membrane and the light chain is responsible for the proteolytic cleavage of VAMP2 (Matteoli et al., 1996; Caleo and Schiavo, 2009). In order to further investigate the role of VAMP2 in the spontaneous form of release, DIV 7 hippocampal neurons were either transfected with the fusion protein TeNT-Lc-EGFP or with a proteolitically inactive mutant TeNT-Lc mut-EGFP, which served as a control (see Materials and methods) (Galli et al., 1994).

It was first important to ensure that the proteolytic activity of TeNT-Lc was maintained when fused to EGFP. In these experiments, FM4-64 was used to assess the function of the presynaptic terminal. DIV 14 hippocampal neurons expressing either construct were depolarised for 90s with 60mM KCl solution containing FM4-64 to load synaptic boutons. The neurons were then fixed and imaged. Synapses expressing TeNT-Lc-EGFP showed a strong reduction in FM4-64 staining whilst its mutant form, TeNT-Lc mut-EGFP, co-localised with well-labeled FM4-64 puncta (Fig. 4.11a). Overall, there was a 2.5 fold reduction in FM4-64 fluorescence intensity when comparing synapses expressing TeNT-Lc-EGFP to those expressing TeNT-Lc mut-EGFP ($P < 0.001$) (Fig. 4.11b) and most of the remaining FM4-64 fluorescence measured for TeNT-Lc expressing neurons is likely due to background labelling. This result confirms that TeNT-Lc-EGFP can block activity-dependent vesicle fusion, despite the EGFP tag.

To determine whether TeNT-Lc affects spontaneous release in the same way as evoked neurotransmitter release, DIV 7 hippocampal neurons were co-transfected with sypHy and TeNT-Lc fused to the red fluorescent protein, Tandem-dimer Tomato (TeNT-Lc-TdT). DIV 14 hippocampal neurons showed clear co-localisation of the presynaptic marker sypHy and TeNT-Lc-TdT (Fig. 4.12a). Control neurons expressing sypHy alone were stimulated with 40 action potentials at 20 Hz, a stimulation that mobilises the readily releasable pool, and showed a measurable increase in sypHy fluorescence (Fig. 4.12bi). However, no change in sypHy fluorescence occurred in neurons expressing TeNT-Lc-TdT (Fig. 4.12bii); also apparent in the mean values for both conditions (control, 0.20 ± 0.034; TeNT-Lc, 0.01 ± 0.007, Fig. 4.12c). Therefore, TeNT-Lc-TdT can also block evoked neurotransmitter release.

In sister cultures, time-lapse imaging at 37°C was performed on control neurons expressing sypHy alone and in neurons co-expressing sypHy and TeNT-Lc-TdT, to measure spontaneous vesicle fusion. Synapses were imaged every 15 seconds for 20 minutes using a confocal microscope with a humidified chamber. The addition of

bafilomycin after 5 min (Fig. 4.12di, black bar below the graph) resulted in an exponential increase in sypHy fluorescence with a time constant of 4.8 min for the control condition (Fig. 4.12di and 4.12de), whereas neurons expressing TeNT-Lc-TdT showed a slower increase in sypHy fluorescence, with a time constant of 26 min (Fig. 4.12dii and 4.12de). The gray traces (in graph fig. 4.12di and 4.12dii) represent the change of sypHy fluorescence normalized to its maximum value and the black trace is the average of all of these synapses (n = 209 synapses from 13 TeNT-Lc mut-EGFP expressing neurons and n = 113 synapses in 7 TeNT-Lc-EGFP expressing neurons).

All together, these results show that the fusion of TeNT-Lc with either EGFP or Tandem-dimer Tomato does not affect its proteolytic activity and this is demonstrated by the inhibition of evoked neurotransmitter release. This result is consistent with previous work where full length tetanus toxin (TeTx) was added to the medium (Schiavo et al., 1992; Matteoli et al., 1996). Whereas evoked release is abolished, spontaneous release is only reduced, suggesting a partial role for VAMP2 in the latter process. Importantly, these results confirmed the validity of the alkaline trap method. When adding bafilomycin to synapses, the gradual increase in sypHy fluorescence is generally taken to reflect vesicle alkalinisation due to exoctyosis. However, it is also possible that during the 15 min bafilomycin treatment, vesicles spontaneously alkalinise without having fused with the plasma membrane, which would also cause an increase in fluorescence. The strong inhibition of the sypHy response suggests that any spontaneous alkalinisation occurs with a much slower time-constant than that measured for vesicle fusion.

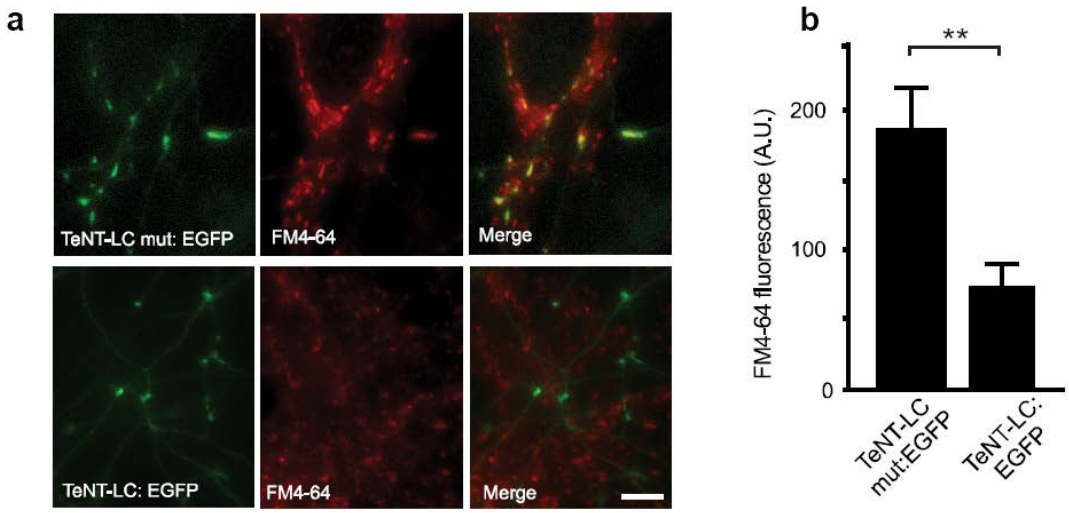


Figure 4.11: Proteolytic activity of TeNT-Lc-EGFP is maintained. (**a**) Hippocampal neurons were transfected with an inactive mutant form of TeNT (TeNT-Lc mut-EGFP; top row) and the functional form of TeNT-Lc fused to EGFP (TeNT-Lc-EGFP; bottom row). Presynaptic activity was assessed using FM4-64. No FM4-64 labeling was found in neurons expressing TeNT-Lc-EGFP compared with TeNT-Lc mut-EGFP (middle column). EGFP and FM4-64 images are merged in the right column. Scale bar = 5µm. (**b**) The graph shows the fluorescence intensity of synapses labeled with FM4-64 for each conditions. Mean values ± s.e.m of n = 209 synapses from 13 TeNT-Lc mut-EGFP expressing neurons and n = 113 synapses in 7 TeNT-Lc-EGFP expressing neurons are shown. Statistical analysis was performed using a Mann-Witney test ($P < 0.001$, **). A.U., Arbitrary units.

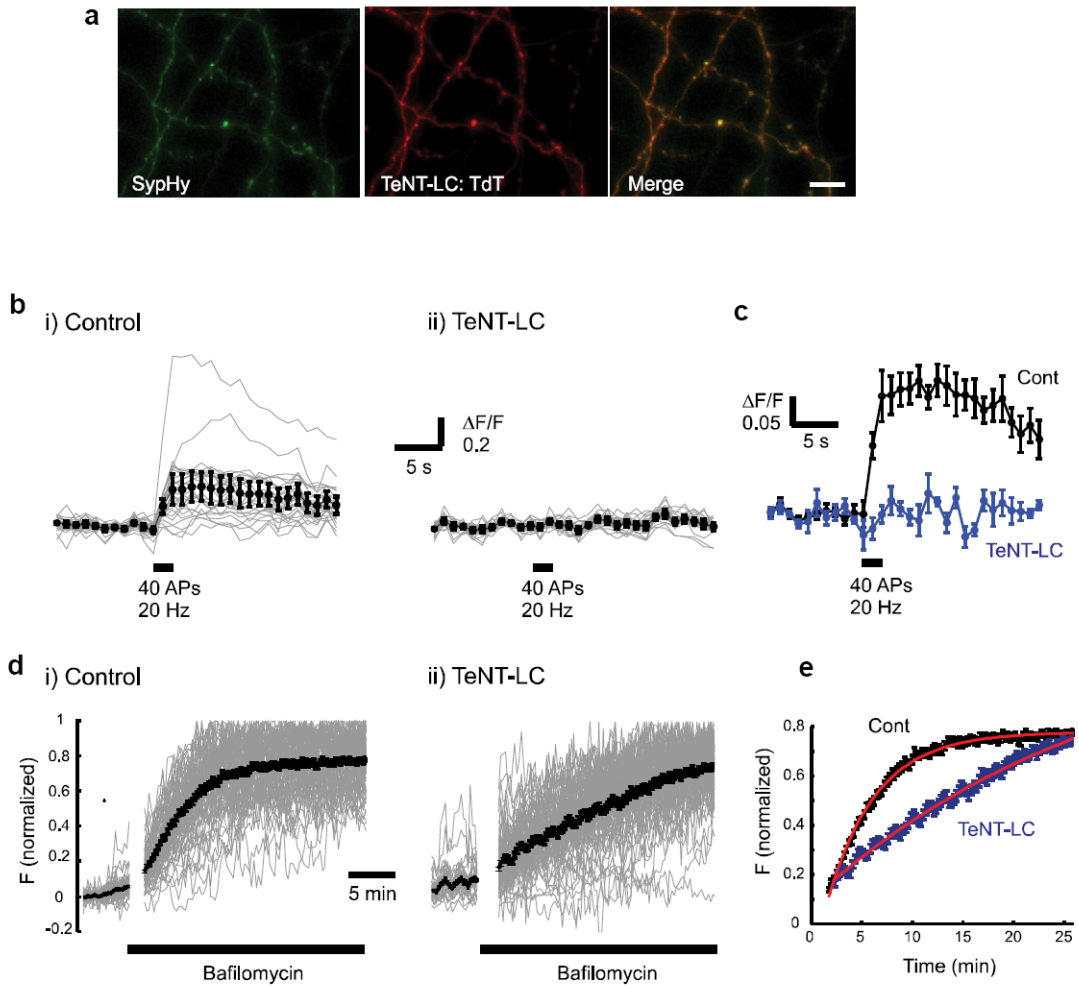


Figure 4.12: Spontaneous vesicle fusion is reduced while evoked release is abolished by TeNT. (**a**) Hippocampal neurons were co-transfected with sypHy (green) and TeNT-Lc-TdT (red). Scale bar = 5µm. (**b**) Change in sypHy fluorescence for control (**i**) and TeNT-Lc expressing (**ii**) neurons in response to 40 action potentials at 20 Hz (black bar). Traces representing sypHy responses of a single synapse (gray) and the average response (black). (**c**) Mean change in fluorescence for control neurons (black trace, n = 5 cells) and neurons expressing TeNT-Lc (blue trace, n = 4 cells) in response to 40 action potential at 20 Hz. (**d**) Graphs show spontaneous increase in sypHy fluorescence after addition of bafilomycin (black bar) for control (**i**) and TeNT-Lc expressing (**ii**) neurons. Traces representing the change in sypHy fluorescence for individual synapses (grey traces) with the average (black trace) overlaid. The fluorescence is normalized to the maximum value for each trace. (**e**) Plot showing an overlay of the change in fluorescence after bafilomycin treatment for control (black) and TeNT-Lc expressing (blue) neurons. Bafilomycin was added at time point zero. Data are the same as that shown in (d). The curves were fit with a single exponential function (red), from which the time constant were established for each condition (control τ = 4.8 min; TeNT-Lc τ = 26 min).

4.7- Vesicles expressing VAMP7 do not undergo vesicle cycling

It has recently been shown that vesicles containing VAMP7 also known as TI-VAMP (Tetanus neurotoxin-Insensitive VAMP), a member of the v-SNARE family, undergo spontaneous vesicle cycling at mature presynaptic terminal (Hua et al., 2011b). In order to verify whether indeed vesicles expressing VAMP7 do recycle, a construct encoding BAP tagged VAMP7 in its C terminal (luminal domain) was generated. VAMP7-BAP expression was first tested on cell lines. CHO cells were co-transfected with VAMP7-BAP and BirA, biotin was added in excess (100µM) to the culture media to ensure that it would not be a limiting factor in the biotinylation reaction of VAMP7-BAP. The detection of biotinylated VAMP7 was done the following day. Transfected CHO cells were initially washed with PBS to remove the excess of biotin and then incubated for 5 minutes in PBS containing strep488 to label biotinylated VAMP7. This resulted in a strong labeling (green, in Fig. 4.13a). This result indicates that the biotinylation reaction of VAMP7-BAP can be detected in live cells using fluorescent streptavidin conjugates.

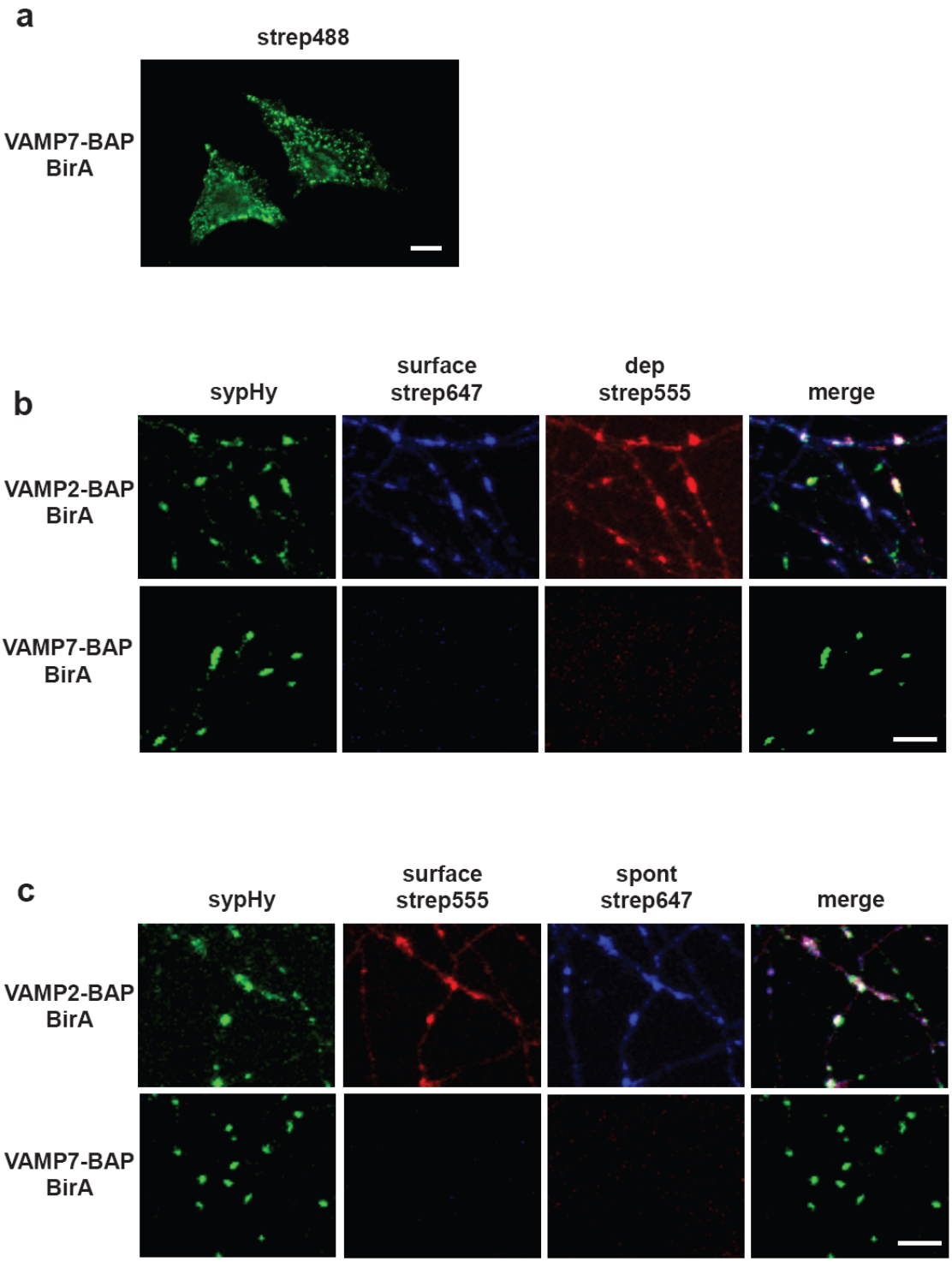
Next, the ability of vesicles expressing VAMP7 to recycle in response to activity or at rest was verified. Sister cultures of hippocampal neurons were transfected at DIV 7 with the presynaptic reporter sypHy and with either VAMP2-BAP/BirA or VAMP7-BAP/BirA. In these experiments biosyn was used as a positive control. At DIV 14 when synapses are mature, one set of neurons had their surface proteins quenched with strep647. After washing excess of strep647, neurons were depolarized in the presence of strep555 to label the recycling pool (Fig. 4.13b). While the surface and the evoked pool were strongly labeled for neurons expressing biosyn (VAMP2-BAP), neither surface nor evoked vesicle fusion events could be detected in neurons transfected with VAMP7-BAP/BirA.

The other set of neurons had their surface proteins labeled with strep555. After washing the excess of strep555, neurons were left for 15 minutes with strep647 to label the spontaneous pool (Fig. 4.13c). Similarly as for the condition above, surface biosyn and the spontaneous pool showed a strong labeling whereas no labeling of biotinylated VAMP7 was found at the surface or of spontaneous vesicle fusion events in neurons expressing VAMP7-BAP/BirA.

All together, these results demonstrate that VAMP7 does not recycle neither in response to activity nor spontaneously. These results are in agreement with Ramirez et al

(Ramirez et al., 2012) and contradict the findings of Edwards and colleagues (Hua et al., 2011b) who showed a role for VAMP7 in spontaneous vesicle recycling.

Figure 4.13: Vesicles expressing VAMP7 do no recycle: (**a**) CHO cells expressing VAMP7-BAP and BirA showed a strong labelling in green after 5 min exposure to PBS in the presence of strep488. In (**b**) and (**c**) Sister cultures of hippocampal neurons were co-transfected with sypHy and with either VAMP2-BAP/BirA or with VAMP7-BAP/BirA. Images show individual synapses expressing sypHy (green). In (**b**) surface biosyn was labelled with strep647 (blue) and evoked pool of vesicles labelled with strep555 (red). Neither surface nor evoked vesicle fusion event could be detected in neurons expressing VAMP7-BAP/BirA. In (**c**), while surface biosyn was labelled with strep555 and the spontaneous pool of vesicles with strep647, no labelling of biotinylated VAMP7 was found neither in the surface nor spontaneous vesicle fusion events. Scale bar = 5µm.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strep488
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BirA
c
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surface
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merge
VAMP2-BAP
BirA
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BirA

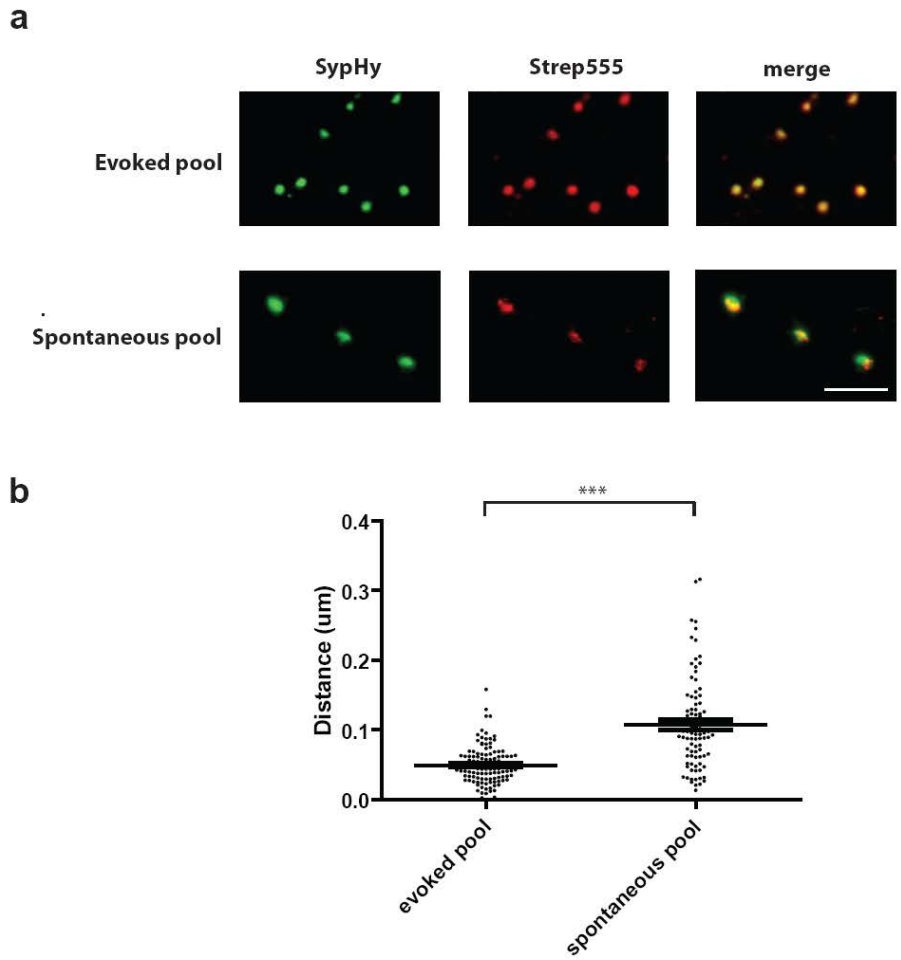
4.8- Localisation of spontaneous and evoked pool of vesicles

Having established that independently-originating (i.e. spontaneous and evoked) vesicle pools coexist at the synapse, it was then important to check whether they shared a common location within the synapse or whether they segregated to distinct compartments. For this purpose, neurons co-expressing sypHy and biosyn were used. SypHy was utilized to label the presynaptic terminal and biosyn to label the evoked and spontaneous pool of vesicles. One set of neurons was depolarized in the presence of strep555 to label the evoked pool of vesicles (Fig. 14a, in red), which showed a perfect co-localisation with sypHy. Another set of neurons was left for 15 minutes at 37°C in the presence of strep555 to label the spontaneous pool of vesicles and revealed a partial localisation with sypHy (Fig. 14a, in red). The centre of strep555 (red) labelling and sypHy (green) was measured as being the point of the highest fluorescence intensity and allowed for the localisation of the presynaptic terminal and pools of vesicles. A measure of the distance between the green (presynaptic terminal) and the red (vesicle pools) was then calculated. Graph 14b plots the calculated distance between the centre of sypHy and strep555 of evoked (n = 119 synapses from 10 neurons) and spontaneous pools (n = 90 synapses from 9 neurons) (see materials and methods for more details on the measurements). The localisation of the evoked pool was significantly different from the spontaneous pool of vesicles at the synapse ($p<0.0001$). While the evoked pool showed a homogenous distribution, the spontaneous pool had a dispersed distribution. Taken together, these results suggest that spontaneous release might occur at different sites than evoked release.

It is important to note that these images were acquired with a confocal microscope using a 100X /1.4 NA (numerical aperture) oil-immersion objective and an excitation wavelength of $\lambda = 543$ nm. Given the Rayleigh criterion, the minimal distance that can be resolved is > 232 nm, which is at the limits of the resolution of sub-compartments within a typical synapse (diameter of about 1 μm). To overcome these limitations and to obtain a better insight into the localisation of the two pools at the synapse, it was important to perform similar experiments using electron microscopy. For this purpose, neurons expressing biosyn had their spontaneous pool labelled with strep488 conjugated to small gold particles (1.4 nm in diameter). To be able to resolve the gold particles they subsequently fixed and silver enhanced (see materials and methods for details on the procedure). Figure 4.14C show three examples of synapses that had their vesicles labelled with gold in response to high potassium treatment. These preliminary results

are encouraging and proof-of-principle that biosyn could be used as a tool to localise vesicles at the ultrastructure level. Unfortunately, due to time restrictions no further data was obtained. Future experiments will use this technique to precisely characterise the distribution of vesicles in presynaptic boutons after exocytosis.

Figure 4.14: Localisation of spontaneous and evoked pool of vesicles. (**a**) Hippocampal neurons were co-transfected with sypHy and biosyn. Images show individual synapses expressing sypHy (green), one set of neurons had their recycling pool of vesicles labelled with strep555 (red) and another set of neurons had their spontaneous pool of vesicles labelled with strep555 (red). Scale bar = 5 µm. (**b**) Graph showing the distance in µm between the highest point of intensity of sypHy and strep555 for the evoked and spontaneous pool. Statistical analysis was performed using a Mann Whitney Test ($P<0.0001$). Values are shown as mean ± s.e.m. (**c**) Three examples of electron microscopy pictures taken from synapses that were labelled with strept488 conjugated to 1.4 nm gold and silver enhanced following spontaneous recycling. Scale bar = 100nm.


a
SypHy
Strep555
merge
Evoked pool
Spontaneous pool
b
***
Distance (um)
0.4
0.3
0.2
0.1
0.0
evoked pool
spontaneous pool
C
Post
Pre

4.9- Vesicular VAMP2 mixes with surface VAMP2 after exocytosis

Our data suggests that the synaptic vesicle VAMP2 is present in three different membrane compartments at the synapse: in vesicles of the recycling pool, in vesicles of the resting pool and on the plasma membrane. This last compartment, is highly enriched with many proteins that have been termed 'synaptic vesicle proteins', but actually show a significant population on the synapse surface and not in vesicles. Indeed, about 20-30% of presynaptic VAMP2 is present on the plasma membrane (Taubenblatt et al., 1999; Sankaranarayanan et al., 2000; Wienisch and Klingauf, 2006). I have previously shown (see chapter IV, paragraph 4.5 and figure 4.10) that the size of the recycling pool correlates with the size of the resting pool suggesting that the levels of spontaneous release may accurately reflect those of evoked release. It would also be interesting to know whether there is any correlation between the size of the evoked or the spontaneous pool of vesicles with that found on the surface. Are they controlled independently of each other or, as suggested by previous findings, could there be significant cross-talk between them?

In order to test this, DIV 14 hippocampal neurons expressing biosyn, first had their surface biosyn labelled with strep647 and were then either depolarized or left for15 minutes at 37°C for spontaneous release to occur in the presence of strep555. The fluorescence intensity of evoked or spontaneous pool was then plotted as a function of the fluorescence intensity of surface VAMP2 (Fig. 4.15a and 4.15b). Each grey dot represents the fluorescence intensity for the evoked (strep555) (Fig. 4.15a) or spontaneous pool (strep555) (Fig. 4.15b) versus VAMP2 surface labelling (strep647) for a single synapse. A tight correlation was found between the size of the evoked or spontaneous pool of vesicles as compared to the surface pool of VAMP2 (Fig. 4.15a and Fig. 4.15b, respectively).

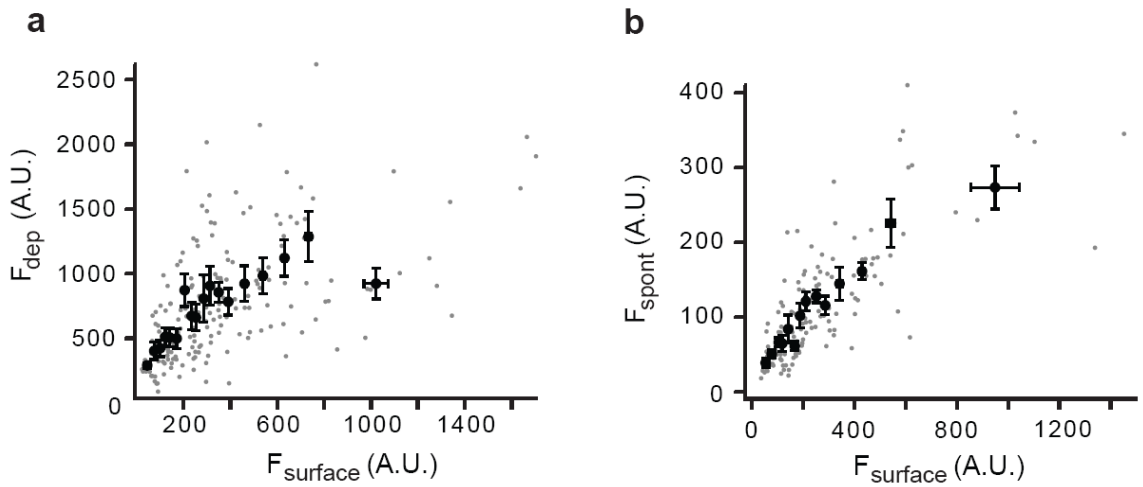


Figure 4.15: Vesicular VAMP2 correlates with surface VAMP2. (**a**) Graph represents a plot of the fluorescence intensity of synapses labelled by depolarization as a function of surface label. Individual synapses are shown in grey and the binned (groups of 10) values in black (n = 445synapses from 20 cells). Correlation coefficient R = 0.55, P< 0.05. (**b**) Graph represents a plot of fluorescence intensity for spontaneously versus surface biosyn for individual synapses (n = 545 synapses from 28 cells). Correlation coefficient R = 0.77, P <0.05.

These results suggest that VAMP2 present on the plasma membrane might arise from protein left over after exocytosis, regardless of the mode of release. If this were the case, mixing between the surface and the vesicular VAMP2 would be expected to occur each time a vesicle fuses, so that after endocytosis some vesicular VAMP2 is left on the surface and some surface VAMP2 is taken up by the vesicle. The alternative to this is that the surface and vesicular VAMP2 are completely independent of each other, so that no mixing occurs during vesicle fusion and the same set of vesicular VAMP2 is endocytosed without any surface VAMP2. In other words, do proteins mix at the surface so that endocytosed vesicles take with it proteins from the surface pool or not?

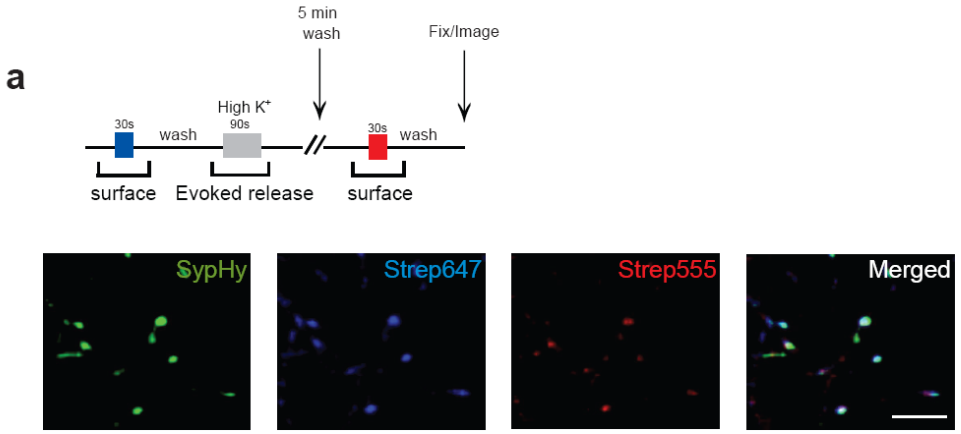
The biosyn technique is ideally suited to resolve this issue. By labelling the surface VAMP2 in one colour, then allowing vesicles to fuse in the absence of extracellular streptavidin and labelling surface VAMP2 in another colour, the degree of mixing between vesicular and surface VAMP2 can be established. At one extreme, if the same set of proteins is endocytosed, without touching the surface pool, then one would expect labelling only with the first colour, but none with the second. Alternatively, any degree of mixing at the membrane would result in labelling with the second colour, as unlabelled biosyn form the newly fused vesicle are left stranded on the surface. Hippocampal neurons co-expressing sypHy and biosyn (where sypHy was only used as

a marker of presynaptic terminals) first had their surface biosyn labelled with strep647 (Fig. 4.16a). Then, in the absence of streptavidin, neurons were depolarized for 90s (Fig. 4.16a). Following this treatment, strep555 was applied to the neurons to label any new biosyn left behind at the plasma membrane after exocytosis. Figure 4.16a shows strong labelling in blue (strep647) and in red (strep555) at the synapses (sypHy), suggesting mixing at the surface. In order to quantify the amount of surface VAMP2 left behind after exocytosis, the same experiment was performed, but this time surface biosyn was first labelled with strep555 (Fig. 4.16b) and, following 90s depolarization in the absence of streptavidin, the newly-deposited biosyn on the plasma membrane was labelled with strep647 (Fig. 4.16b). Quantification of the average fluorescence intensity of strep555 and strep647 before and after stimulation (90s depolarization) at individual synapses is shown in figure 4.16c and 4.16d, respectively. The data obtained for each fluorophore was then normalised to the initial surface biosyn (before any fusion), pooled and plotted in figure 4.16e. The graph shows that about 40% of vesicular biosyn was deposited on the plasma membrane after the entire recycling pool of vesicles had recycled. These results suggest that a substantial mixing occurs between the surface VAMP2 and VAMP2 from the recycling pool.

Similar experiments were performed for spontaneous release. In this case, instead of depolarizing neurons, vesicular exocytosis was allowed to occur spontaneously in the absence of streptavidin (15 minutes at 37°C in HBS containing APV, CNQX, TTX and no Ca^{2+}) (Fig. 4.17a and 4.17b). Once again fluorescent labelling is observed in both wavelengths. Quantification of the average fluorescence intensity of strep555 and strep647, before and after spontaneous vesicle fusion, is shown in figure 4.17c and 4.17d, respectively. Figure 4.17e (pooled data from both fluorophores) shows that about 35% of vesicular biosyn was deposited on the plasma membrane after the entire spontaneous pool of vesicles had recycled. Furthermore, no significant difference was found between the amount of surface mixing following 90s depolarisation, or vesicles fusing spontaneously ($P>0.05$).

Taken together, these results demonstrate that both evoked and spontaneous fusion results in mixing of vesicular and surface VAMP2 molecules at the synapse, suggesting that synaptic vesicles lose their prior molecular identity. This finding is in accordance with previous studies (Fernandez-Alfonso et al., 2006; Wienisch and Klingauf, 2006).

Figure 4.16: Vesicular VAMP2 mixes with the surface VAMP2 following evoked release. Hippocampal neurons co-expressing sypHy and biosyn were treated following different protocols indicated in schematic diagrams. Images show individual synapses expressing sypHy (green). (**a**) Labelling of surface biosyn with strep647 (blue) for 30s. Followed by a depolarization for 90s in the absence of streptavidin, the newly exocytosed biosyn present at the surface are labelled with strep555 (red). (**b**) Labelling of surface biosyn with strep555 (red) for 30s. Followed by a depolarization for 90s in the absence of streptavidin, the newly exocytosed biosyn present at the surface are labelled with strep647 (blue). Scale bar = 5 µm. Graphs (**c**) and (**d**) show the average fluorescence intensity of surface biosyn before and after depolarisation labelled with strep555 (n = 570 synapses from 19 neurons) or strep647 (n = 570 synapses from 19 neurons), respectively. Statistical analysis was performed using a Mann Whitney Test ($P<0.0001$). Values are shown as mean ± s.e.m.


a
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Evoked release
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Strep647
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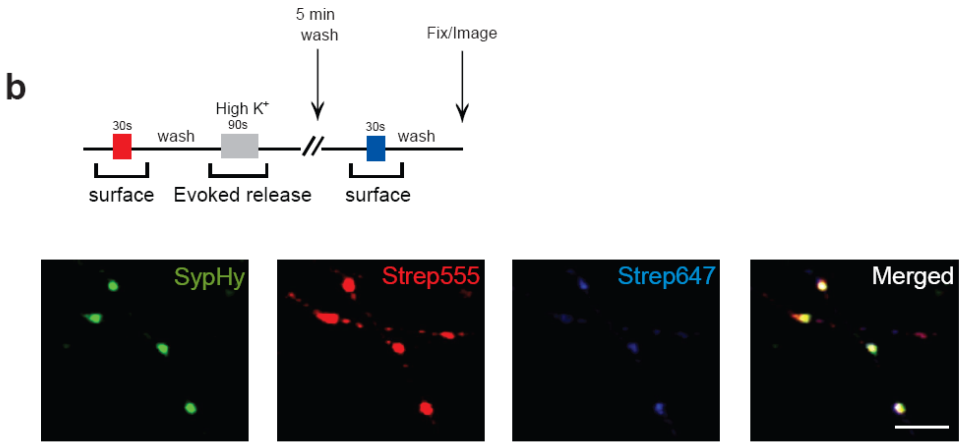
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surface
Evoked release
surface
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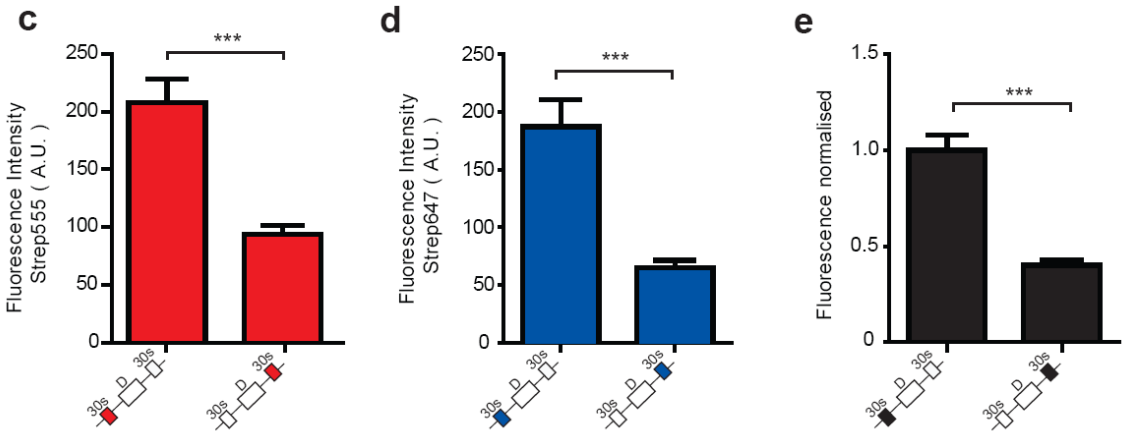
Strep647
Merged


c
Fluorescence Intensity
Strep555 (A.U.)
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0
***
d
Fluorescence Intensity
Strep647 (A.U.)
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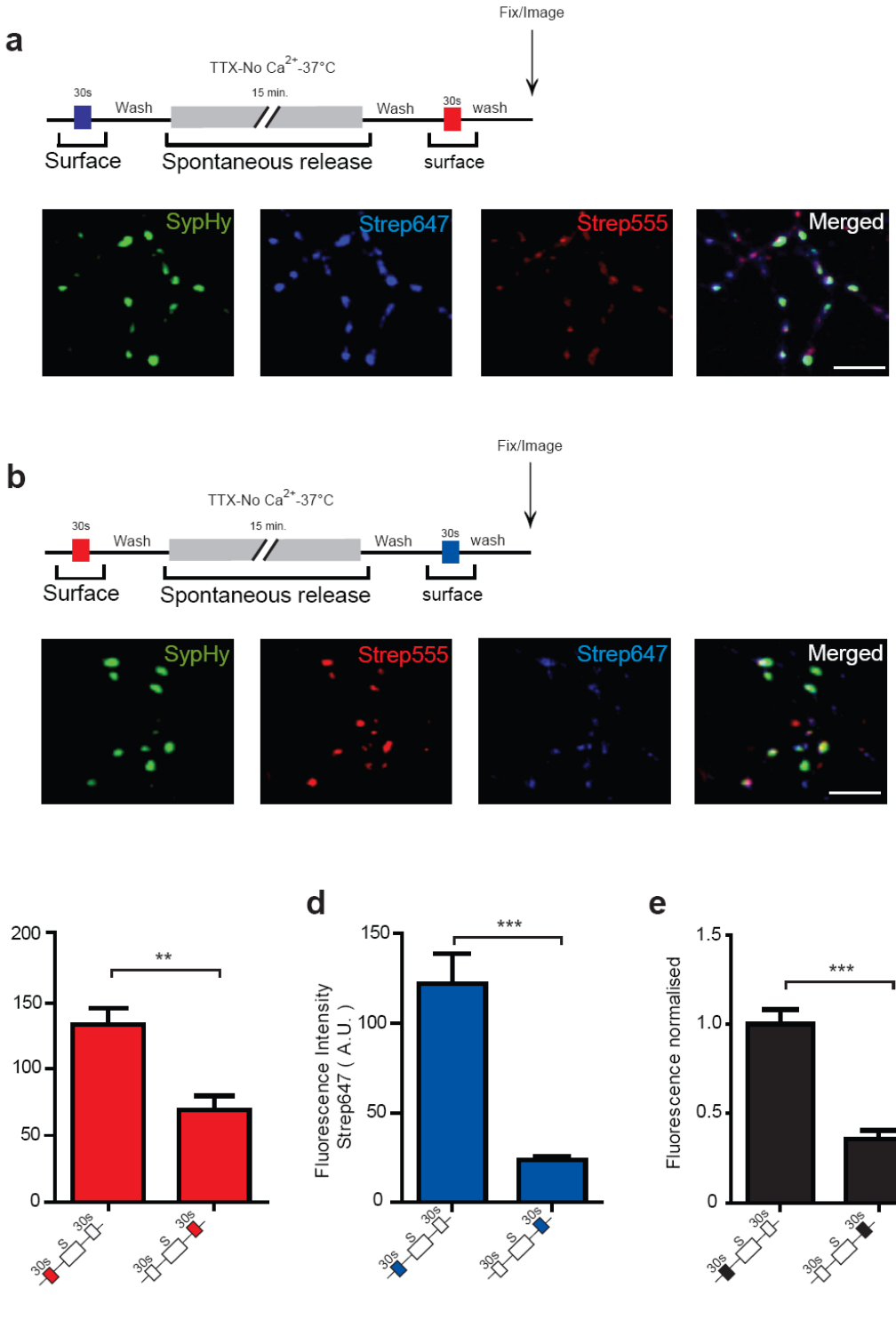
200
150
100
50
0
***
e
Fluorescence normalised
1.5
1.0
0.5
0
***

Figure 4.17: Vesicular VAMP2 mixes with surface VAMP2 following spontaneous release. Hippocampal neurons co-expressing sypHy and biosyn were treated following different protocols indicated in schematic diagrams. Images show individual synapses expressing sypHy (green). (**a**) After labelling surface biosyn with strep647 (blue) for 30s, neurons were then left for 15 minutes in the presence of TTX, in the absence of Ca^{2+} and streptavidin, the newly exocytosed biosyn present at the surface were labelled with strep555 (red). (**b**) After labelling surface biosyn with strep555 (red) for 30s, neurons were then left for 15 minutes in the presence of TTX, in the absence of Ca^{2+} and streptavidin, the newly exocytosed biosyn present at the surface were labelled with strep647 (blue). Scale bar = 5 µm. Graphs (**c**) and (**d**) show the average fluorescence intensity of surface biosyn before and after depolarisation labelled with strep555 (n = 570 synapses from 15 neurons) or strep647 (n = 570 synapses from 15 neurons), respectively. Statistical analysis was performed using a Mann Whitney Test (***P<0.0001, P<0.001**). Values are shown as mean ± s.e.m.


a
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TTX-No Ca2+-37°C
30s
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30s
wash
Surface
Spontaneous release
surface
SypHy
Strep647
Strep555
Merged
b
Fix/Image
TTX-No Ca2+-37°C
30s
Wash
15 min.
Wash
30s
wash
Surface
Spontaneous release
surface
SypHy
Strep555
Strep647
Merged
c
Fluorescence Intensity Strep555 (A.U.)
200
150
100
50
0
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d
Fluorescence Intensity Strep647 (A.U.)
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100
50
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e
Fluorescence normalised
1.5
1.0
0.5
0
***

4.10- Discussion

4.10.1- Summary of results

In summary, while biosyn was used effectively to report evoked vesicle fusion (see chapter III), in this chapter it was also used to reliably detect and characterise spontaneous fusion events at the presynaptic terminal. Using different stimulation protocols it was possible to demonstrate the existence of two distinct pools of vesicles that recycle independently, and with different kinetics, at the presynaptic terminal. One pool is mobilized in response to neuronal activity and the other one fuses spontaneously with the plasma membrane. Whereas fusion of activity-dependent vesicles originates from the recycling pool, the resting pool of vesicles, which cannot be mobilized by neuronal activity, is the source for spontaneous vesicle release. Confocal imaging of both forms of release suggested that they may occur at different sites at the synapse. Furthermore, evoked release was abolished when VAMP2 was cleaved by the application of tetanus toxin, whereas spontaneous fusion persisted under the same conditions, suggesting that different molecules might be implicated in the fusion machinery during spontaneous release. Finally, it was demonstrated that vesicular VAMP2 mixes with the existing pool of surface VAMP2 molecules following either evoked or spontaneous fusion of vesicles at the synapse.

4.10.2- Vesicular VAMP2 mixes with surface VAMP2

A fraction of synaptic vesicle proteins are present on the plasma membrane and their relative amounts vary depending upon the presynaptic protein: for instance, 30% of total synaptic VAMP2 is found on the surface (Taubenblatt et al., 1999; Sankaranarayanan et al., 2000; Wienisch and Klingauf, 2006), 8% for synaptophysin (Granseth et al., 2006), 24% for synaptotagmin (Wienisch and Klingauf, 2006) and 2% for the glutamate transporter vGlut1 (Balaji and Ryan, 2007). Although it is unclear why different synaptic vesicle proteins are present at very different levels on the plasma membrane, it is apparent that some are highly enriched (e.g. VAMP2 and synaptotagmin-1). In this study, 30% of biosyn was found to be expressed on the plasma membrane which is consistent with what was previously shown when measuring the surface levels of synaptophluorin and synaptotagmin (Taubenblatt et al., 1999; Sankaranarayanan et al., 2000; Fernandez-Alfonso et al., 2006; Granseth et al., 2006; Wienisch and Klingauf, 2006). Following the release of either the entire recycling pool or the spontaneous pool of vesicles, about 40% of the vesicular biosyn replaced biosyn on the plasma membrane

irrespective of the fusion mode, strongly suggesting that vesicular and surface proteins mix. Similar results have previously been reported using different approaches. Fernandez-Alfonso *et al.* used VAMP2 and synaptotagmin, both of them fused to pHluorin (Fernandez-Alfonso et al., 2006). After bleaching the surface pHluorin and following stimulation, they found that about 50% of the bleached molecules were replaced by unbleached pHluorin molecules. Using a different approach, Wienisch *et al*. inserted a tobacco etch virus (TEV)-protease cleavage sites between VAMP2 or synaptotagmin and the pHluorin domain. The pHluorin present on the surface of the plasma membrane was then removed by exposure to a TEV protease that only digested surface pHluorin molecules. Following stimulation, they found that digested surface molecules were recycled into synaptic vesicles and new undigested molecules appeared at the surface (Wienisch and Klingauf, 2006), once again demonstrating that vesicular proteins mix with proteins already present on the plasma membrane. More interestingly, a correlation was found between the amount of mixing and the stimulus length and frequency. Indeed, a strong stimulation led to a larger amount of mixing between surface and vesicular proteins, whereas little mixing was found for weaker stimuli (Fernandez-Alfonso et al., 2006). In addition, vesicular and membrane protein mixing following spontaneous cycling has also been described. Fernandez-Alfonso *et al*. found that 21% of vesicular synaptotagmin 1 is replaced by surface synaptotagmin 1 when allowed to recycle at rest for 8 minutes at room temperature (Fernandez-Alfonso et al., 2006). In the present study, spontaneous cycling was measured for VAMP2 at 37°C, which could account for the higher level of mixing observed.

In some other reports, mixing was not observed. Gandhi *et al.* used synaptophluorin to image single vesicle fusion events. After bleaching surface pHluorin, they found a complete recovery of the fluorescence following exo-endocytosis of a single vesicle and concluded that a 'kiss-and-run' type of recycling occured (Gandhi and Stevens, 2003). In this mode of release, vesicles open and close transiently without full collapse with the plasma membrane resulting in no vesicular proteins dispersing onto the axonal surface (Gandhi and Stevens, 2003). It has been proposed that 'kiss-and-run' is the predominant form of endocytosis in response to low-frequency stimulus paradigms, whereas clathrin-dependent endocytosis dominates at high frequency stimulus (Aravanis et al., 2003; Gandhi and Stevens, 2003; Fernandez-Alfonso and Ryan, 2004; Harata et al., 2006). However, other studies have used spH- or GFP-tagged VAMP2, to show that exocytosis is accompanied by the dispersion of synaptic vesicle proteins from the presynaptic

bouton onto the perisynaptic axonal surface, even for weaker stimulations (Sankaranarayanan et al., 2000). Moreover, during sustained strong stimulation, newly exocytosed vesicular VAMP2 on the plasma membrane can spread laterally so that it mixes with neighbouring synapses (Li and Murthy, 2001). This agrees with the notion that following full collapse of the vesicle, vesicular proteins deposited onto the active zone plasma membrane organise into 'raft-like patches' which then diffuse along the surface to a different compartment for later compensatory endocytosis (Wienisch and Klingauf, 2006). It has recently been proposed that these vesicular proteins present on the plasma membrane could correspond to a "readily retrievable pool" of preassembled vesicle proteins that are preferentially retrieved upon exocytosis (Wienisch and Klingauf, 2006; Hua et al., 2011a).

In conclusion, a substantial amount of mixing exists between vesicular and surface proteins following exo-endocytosis. Although more experiments are needed to understand the role of these proteins on the plasma membrane, our findings are also a warning for studying vesicle trafficking using synaptic vesicle proteins tags. When studying vesicle cycling it is important to take into account the surface fraction of vesicular proteins to avoid erroneous conclusions.

4.10.3- Presynaptic pools of vesicles

The main aim of this chapter has been to investigate whether spontaneous and evoked release arise from the same pool of vesicles or if they draw from two independent pools at the presynaptic terminal. A new tool 'biosyn' was used to resolve this question. Biosyn has been previously shown to effectively label vesicles that fuse in response to neuronal activity (see Chapter III). In this current chapter, spontaneous release was also shown to be detected using the same tool. One of the main advantages of using biosyn is that subsets of vesicles can be labelled sequentially, with different fluorophores, in the same experiment. Taking advantage of this useful property, biosyn technology has permitted us to demonstrate and characterize the existence of two independent pools of vesicles supporting spontaneous and evoked release. One pool of vesicles was mobilized in response to neuronal activity, and originated from the recycling pool, which is formed by the rapidly releasable pool (RRP) and the reserve pool (Sudhof, 2004). On the other hand, spontaneous release is supported by vesicles originating from the resting pool which was previously defined as being refractory to neuronal activity (Sudhof, 2004). This finding is controversial as a number of recent studies have found evidence for and against two independent pools of vesicles supporting evoked and

spontaneous neurotransmitter release. Furthermore, until now, the role of the resting pool of vesicles has not been well understood.

4.10.3.1- Two independent pools of vesicles for spontaneous and evoked release

Sara *et al.* were the first to propose that spontaneous and evoked release is supported by distinct vesicle pools that recycle independently in dissociated hippocampal cultures (Sara et al., 2005). Using FM dyes they showed that vesicles fusing spontaneously with the plasma membrane were preferentially recycled for subsequent spontaneous release rather than evoked release. In addition, whole-cell recordings of mEPSCs showed that inhibition of neurotransmitter refilling of spontaneously recycled vesicles with the vacuolar ATPase inhibitor folimycin, resulted in a decrease in mEPSC frequency, while evoked release was only slightly affected, further confirming that vesicles recycling spontaneously originate from a different pool of vesicles than the ones recycling in response to neuronal activity.

Two separate pools of vesicles supporting either evoked or spontaneous release were also described in inhibitory presynaptic terminals (Mathew et al., 2008). Indeed, when inhibitory synapses were loaded by stimulation with FM1-43 and then unloaded with activity in the absence or presence of kainate, they found that kainate increased the rate of FM1-43 destaining. The same result was obtained when the dye was taken up spontaneously and then unloaded spontaneously in the presence or absence of kainate. However, when inhibitory neurons were loaded with FM1-43 spontaneously and subsequently unloaded by stimulation, they found that kainate did not affect FM1-43 destaining rate, suggesting that spontaneous and evoked release could be differentially modulated by kainate and that these two forms of release may originate from distinct vesicle pools (Mathew et al., 2008).

The finding that independent pools of vesicles are at the origin of spontaneous and evoked forms of release at the presynaptic terminal remains controversial as a number of recent studies have provided contradictory results (Groemer and Klingauf, 2007; Wilhelm et al., 2010; Hua et al., 2011a).

Using the same optical imaging techniques as Sara *et al.* (Sara et al., 2005), Groemer *et al.* have reached different conclusions. Indeed, following the loading of two spectrally distinct FM dyes, one loaded spontaneously and the other in response to activity, at the same bouton, they found that the destaining rate profiles for both labelling conditions were identical, suggesting that both fusion events originated from the same pool of

vesicles (Groemer and Klingauf, 2007). The reason for this difference in the outcome of apparently similar experiments is not clear. However, the labeling of spontaneous release in the Groemer *et al.* study necessitated long exposure of the neuronal cultures to FM dye which could increase the background fluorescence and non-specific labeling, rendering analysis more complex and possibly leading to false interpretation of the nature of the recycling events (see Chapter III for discussion).

More recently, a study describing a new pH-sensitive reporter, cypHer5E, coupled to either a monovalent streptavidin (cypher-streptavidin), which binds to biosyn or to an antibody directed against the luminal domain of synaptotagmin1 (αSyt1-cypHer) was used to follow labeled vesicles after many rounds of release (Hua et al., 2010). Using a two-step protocol, Hua *et al.* first labeled vesicles either in response to stimulation or spontaneously. They found that following a subsequent stimulation, both activity and spontaneously loaded vesicles, had similar release kinetics and concluded that both forms of release draw upon the same pool (Hua et al., 2010). However, this study did not take into account the presence of a large pool of vesicular proteins (such as Vamp-2 and synaptotagmin1) on the presynaptic plasma membrane. Indeed, this chapter as well as other studies have clearly shown that vesicular and surface proteins mix following evoked or spontaneous release (Fernandez-Alfonso et al., 2006; Wienisch and Klingauf, 2006) (see discussion paragraph 4.9.2). Thus, the labeling protocol used to study vesicle cycling will mark both surface and vesicular proteins, leading to non-specific labeling. This renders the study of vesicle pools impossible unless the plasma membrane pool of vesilce proteins are taken into account. Accordingly, recent work in the lab has confirmed that surface proteins do contribute to the signal measured, rendering any conclusions invalid. Stimulation with 600 APs at 20 Hz after labeling the surface pool of Syt1 with αSyt1-cypHer led to a fluorescence response similar to the one observed after labeling the evoked pool vesicles, indicating that recycling vesicles take up surface and vesicle proteins alike (Andreae et al., 2012). In other words, any surface labeling during spontaneous mobilization will result in changes in fluorescence in response to activity that do not necessarily involve spontaneous vesicles.

Another recent study by Wilhelm *et al*, has also addressed the question of whether one or two pools of vesicles are at the origin of evoked and spontaneous release (Wilhelm et al., 2010). One set of experiments were based on the use of a biotinylated synaptotagmin1 antibody (biotinylated α-Syt1) to label vesicles spontaneously (15 min at RT) or following stimulation (600AP at 20Hz). They then added a fluorescent streptavidin to the extracellular medium and mobilized vesicles once again, either

spontaneously or following stimulation. If a vesicle loaded with the biotinylated antibody undergoes another round of exocytosis, it will then bind to the streptavidin probe. They found the same amount of fluorescence labeling regardless of the loading sequence (spontaneous or evoked) with either biotinylated α-Syt1 or the fluorescent streptavidin, leading to the conclusion that evoked and spontaneous release arise from the same pool of vesicles. In fact, they found that similar levels of staining were observed in all conditions. This is a strange finding as we know that spontaneous release does not mobilize as many vesicles as the entire recycling pool, so differences in staining should be observed (see figure 4.4). In addition, the authors did not account for surface synaptotagmin-1 staining. Along similar lines, the authors also used two different versions of syt-1 antibodies that recognize different extracellular epitopes. In combination with high resolution STED imaging, they also concluded that individual vesicles could be stained with both antibodies delivered sequentially by activity and spontaneous release. A major concern with this approach is that no controls were done to check the saturation of each antibody after labeling. This entire set of experiments lack the appropriate controls.

In another set of experiments, they used synaptopHluorin (spH) to report exocytosis. They first labeled the activity-dependent pool in the presence of folimycin, a blocker of vesicle reacidification. Next, they allowed exocytosis of the activity-dependent pool to occur either following a second stimulation or at rest (15 min at RT) in the presence of bromophenol blue to quench surface spH, but without folimycin. They found similar levels of exocytosis whether vesicles recycled spontaneously or in response to stimulation and concluded that the same pool of vesicles is at the origin of both forms of release. The major pitfall in this experiment is that the removal of folimycin could result in the reacidification of the vesicles independently from recycling and produce a decrease in spH fluorescence at rest (Sankaranarayanan and Ryan, 2001). Definitive conclusion cannot be drawn from these experiments unless appropriate controls are performed.

4.10.3.2- The resting pool recycles

At the presynaptic terminal vesicles are organized in three distinct pools: the readily releasable pool (RRP), the reserve pool and the resting pool (Sudhof, 2004). The first two pools form the recycling pool and are recruited in response to neuronal activity. The resting pool of vesicles is refractory to neuronal activity and its function is largely unknown. In this chapter, the use of sypHy has allowed us to demonstrate that vesicles

released spontaneously originate from the resting pool. No studies had hitherto demonstrated the origin of the spontaneous pool. However, some studies have shown that a set of vesicles that are thought to represent the resting pool could be mobilized in response neuronal activity. For example, Ikeda *et al.* demonstrated that a mild stimulation of 0.2 Hz for 10 min did release vesicles from the resting pool in hippocampal synapses (Ikeda and Bekkers, 2009). Nonetheless, it is possible that the activity they observed actually corresponded to spontaneous release due to the low rate of stimulation used and its long duration. Similarly, Poskanzer *et al.* described a resting pool of vesicles at the Drosophila neuromuscular junction that can be mobilized during evoked stimulation. However, this occurred only in the case of endocytic blockade (Poskanzer and Davis, 2004). It has recently been shown that the size of the resting pool is defined by the equilibrium between the cyclin-dependent kinase 5 (CDK5) and calcineurin (CN) activity (Kim and Ryan, 2010). When CDK5 is inhibited, vesicles from the resting pool become competent for recycling. On the other hand, when CN is inhibited, the size of the resting pool is increased, presumably by drawing from the recycling pool of vesicles. (Kim and Ryan, 2010). It thus appears that the resting pool can participate in vesicle cycling in specific circumstances and that finely regulated exchanges can occur between the resting and recycling pools.

4.10.4- Different molecular requirement for spontaneous and evoked release

As evidence accumulates on the existence of two pools of vesicles, it will important to establish what the molecular partners of these pools are. In fact, a number of molecules of the release machinery have been shown to participate in the regulation of either spontaneous or evoked release.

In this chapter, it was shown that VAMP2 cleavage by tetanus toxin light chain (TeNT-Lc) abolished evoked release while spontaneous release was only reduced. Similar results were found at the crayfish neuromuscular junction (Hua et al., 1998). Indeed, following TeNT-Lc and botulinum toxin B- Lc (BoNT/B-Lc) treatment, evoked release was blocked while spontaneous release was only slightly affected. In mice and Drosophila lacking the SNARE protein VAMP2, evoked release was also abolished while spontaneous release persisted but at a lower frequency (Deitcher et al., 1998; Schoch et al., 2001). All together, these findings suggest that spontaneous release may be less reliant on VAMP2 function than evoked release. It may also suggest that different VAMP variants are implicated in evoked and spontaneous release, which leaves open the question of what VAMP isoform is needed for spontaneous fusion. A

recent study has actually addressed this question and found that VAMP7 or TI-VAMP (Tetanus neurotoxin insensitive-vesicle associated membrane protein) may be involved in spontaneous vesicle recycling (Hua et al., 2011b). However, these finding do not agree with the results presented in this chapter. We observed neither evoked nor spontaneous release using a biotinylated version of VAMP7. Similar results were also recently obtained by Kavalali and colleagues with VAMP7 fused to pHluorin (Ramirez et al., 2012). Instead, these authors identified another non-canonical SNARE protein Vps10p-tail-interactor 1a (Vti1a), previously thought to participate in the endosomal pathway, as being a molecular maker for vesicles fusing spontaneously (Ramirez et al., 2012). More importantly, they show that knock down of Vti1a selectively reduces spontaneous release, leaving evoked release unperturbed, suggesting it is a vital component of the release machinery for spontaneous vesicle fusion.

Other molecular components of the release machinery appear to be differentially implicated in the regulation of evoked and spontaneous fusion. In mice lacking the SNARE protein SNAP-25 (Washbourne et al., 2002; Bronk et al., 2007) and in Drosophila lacking syntaxin1a (Schulze et al., 1995), evoked release was drastically diminished whereas spontaneous release still occurred, suggesting that different t-SNAREs might be implicated in the two forms of release. In other cases, evoked release is unchanged while spontaneous release is altered. For example, over-expression of synaptotagmin-12, a synaptotagmin isoform that does not bind Ca^{2+}, in hippocampal neurons resulted in an increase in spontaneous release, while evoked release remained unaltered (Maximov et al., 2007). In other studies, modifications of the molecular machinery that resulted in a decrease in evoked release were accompanied by an increase in spontaneous release. For instance, in neurons lacking synaptotagmin-1 (Syt1), a Ca^{2+} sensor for synchronous release, a loss of evoked release and an increase in spontaneous release were observed (DiAntonio et al., 1993; Littleton et al., 1994) (Maximov and Sudhof, 2005). The implication of Syt1 as a calcium sensor regulating spontaneous release is debated. Instead, the double C2 domain 2b protein (Doc2b) has been suggested as an alternative high-affinity Ca^{2+} sensor for spontaneous release (Groffen et al., 2010). Similar to Syt1, complexins 1 and 2 knockdown resulted in an increase spontaneous release paired with a decrease in evoked release (Maximov et al., 2009). A further study by Huntwork *et al.* has suggested a role of complexin as a fusion clamp for spontaneous release (Huntwork and Littleton, 2007).

Finally, differences could also exist in the endocytic machinery that retrieves synaptic vesicles after evoked and spontaneous release. A study by Kavalali *et al.* has recently shown that synchronous release originated from the recycling pool and was dependent on the endocytic protein dynamin whereas spontaneous release originated from a pool that was independent of dynamin activity (Chung et al., 2010).

All together, these findings clearly suggest that the regulation of spontaneous and evoked release may require distinct molecules. However, the molecular identity of the corresponding pools of vesicles is not known.

4.10.5- Localization

A spatial segregation of evoked and spontaneous release has been described in the giant presynaptic terminal of goldfish retinal bipolar cell. Imaging individual vesicle fusion events using TIRF imaging showed that evoked release occurred at the ribbon (the active zone in these type of sensory synapses), whereas spontaneous release events happens at extra-ribbon sites (Zenisek, 2008). In agreement with this, in this chapter, the evoked pool of vesicles was found to co-localise with the presynaptic marker sypHy while the spontaneous pool of vesicles had a heterogeneous distribution across synapses and on average seemed to have a peri-synaptic localisation. This spatial segregation may account for the recent evidence that evoked and spontaneous release activate different sets of postsynaptic NMDA and AMPA receptors (Atasoy et al., 2008; Sara et al., 2011). The latter finding suggests that evoked and spontaneous neurotransmitter release might lead to the activation of different signalling pathways in the postsynaptic neuron. Further investigation into the localisation of evoked versus spontaneous fusion events are now required.

CHAPTER V

Vesicle cycling in a developing neuron

5.1- Introduction

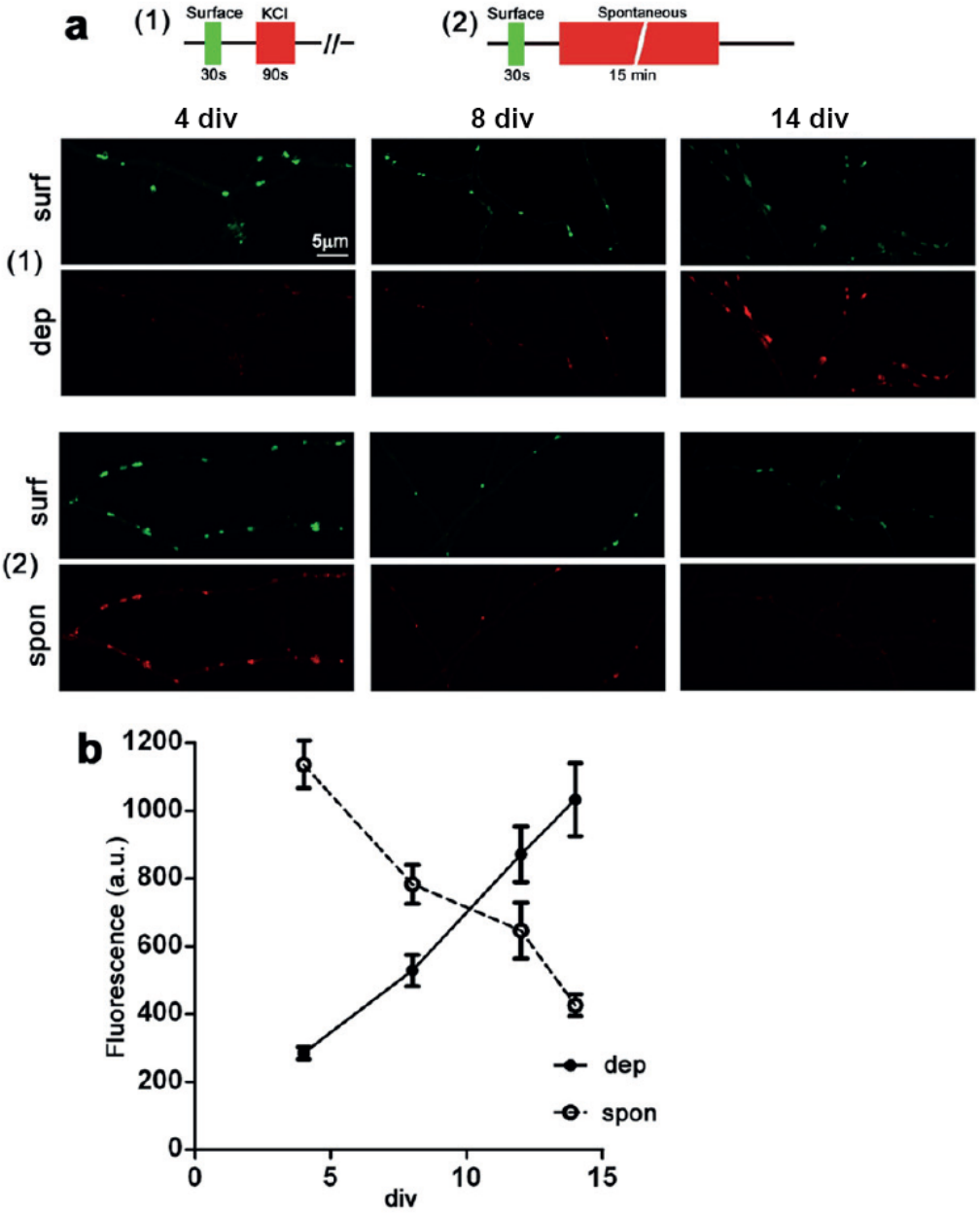
During nervous system development, neurons project axons that elongate to reach their destination via their growth cone, before branching and interconnecting with other neurons to form functional synapses. In the previous chapter, biosyn was shown to specifically label evoked and spontaneous vesicle cycling in mature neurons, whilst also allowing the characterization of their pool of origin. The formation of functional synapses requires clustering of different pools of vesicles at active zones and the subsequent mobilization of vesicles to release neurotransmitter. While many studies have focused on the functional aspects of vesicle cycling at mature presynaptic terminals, much less is known about this process in development, during the period of synapse formation. The few studies that have been performed early in development have clearly shown that both spontaneous and activity-dependent recycling does occur in the growth cone (Diefenbach et al., 1999) and along the growing axons prior to synapse formation (Matteoli et al., 1992; Kraszewski et al., 1995; Dai and Peng, 1996; Nakata et al., 1998; Zakharenko et al., 1999; Sabo et al., 2006). In fact, early work performed in xenopus cholinergic motor neurons showed that the release of acetylcholine occurred constitutively along an axon, even in the absence of a postsynaptic partner (Zakharenko et al., 1999; Zakharenko and Popov, 2000). Further still, experiments that used 'sniffer' cells expressing acetylcholine receptors showed that neurotransmitter release could be elicited by stimulating the neuron in presence of extracellular Ca^{2+} (Zakharenko et al., 1999). Together, these findings strongly suggest that there exists a pool of vesicles that is capable of cycling and releasing neurotransmitter in immature axons. This is further emphasized by electron microscopy images of growing axons that show clusters of vesicles present all along an axon. Although these vesicle clusters may simply be part of the transport mechanism that shuttles vesicles to their final destination, it is also possible that they fuse with the plasma membrane along the way and may therefore contribute to neurotransmitter release (Kraszewski et al., 1995; Ahmari et al., 2000). Indeed, it is now well established

that vesicle clusters are transported along the axon as part of pre-assembled packets of presynaptic components, which are recruited upon contact with a postsynaptic partner (Kraszewski et al., 1995; Ahmari et al., 2000; Friedman et al., 2000; Okabe et al., 2001). At the site of synaptic contact, the number of synaptic vesicles increases and progressively organises into the characteristic different pools of vesicles (Dyson and Jones, 1980; Blue and Parnavelas, 1983; Mozhayeva et al., 2002). This is probably best exemplified in a detailed study that followed the functional and structural maturation of the presynaptic terminal, focusing on the evolution of activity-dependent release (Mozhayeva et al., 2002). The authors found that vesicles would initially show a reluctant form of vesicle recycling, that they interpreted as the presence of a recycling pool that could only be recruited in response to strong stimulation. Following this, the putative presynaptic terminal incorporated a faster form of vesicle cycling that the authors attributed to the presence of a readily releasable pool (RRP). Finally, following the appearance of the RRP, a reserve pool became apparent that allowed the active zone to release in response to sustained stimulation (Mozhayeva et al., 2002). It is important to note that this study did not consider spontaneous release, focusing exclusively on evoked release. However, other studies did follow spontaneous release during development, using a combination of fluorescent tools (mainly fluorescently tagged antibodies) to label vesicle cycling. They found that although young neurons (DIV 3) showed robust spontaneous cycling, this form of release was gradually down-regulated (Kraszewski et al., 1995; Coco et al., 1998). Until now, there has been no complete study of the different pools of vesicles and their modes of release during neuron development. One possible reason for this is the lack of adequate tools to simultaneously follow both forms of release in growing axons.

The aim of this chapter is to study how VAMP2 positive vesicle cycling occurs during development, with particular emphasis on the distinct modes of release described in the previous chapter. Do spontaneous and evoked forms of release occur simultaneously throughout development? Do they share vesicles early on? As shown for mature synapses, biosyn and synpatophysin -pHluorin are ideal tools to study spontaneous versus evoked release and, in this chapter, they will be exploited to follow vesicle cycling in developing hippocampal neuron.

5.2- Developmental switch in vesicle cycling modes

In order to investigate the degree of involvement of evoked versus spontaneous vesicle cycling during development, hippocampal neurons expressing biosyn at different developmental stages were used. 4, 8, 12 and 14 days *in vitro* (DIV) neurons were first incubated for 30s in HBS containing strep488 to quench the surface biosyn. One set of neurons was then depolarized with a high potassium solution containing strep555 for 90s to label the activity dependant vesicles cycling pool, and another set of neurons was left for 15 minutes, at 37°C, in HBS lacking Ca^{2+}, but in the presence of TTX and strep555 to label vesicles cycling spontaneously. Figure 5.1a and 1b, clearly show that early in development (4 DIV) spontaneous labeling is predominant, while minimum levels of evoked release take place. However, in complete contrast, high level of evoked labeling was seen in mature neurons (14 DIV) with low levels of spontaneous release. This result demonstrates that a developmental switch in the preferred mode of vesicle cycling occurs in hippocampal neurons, from mainly spontaneous release early in development to a mostly evoked form of release in mature neurons.



Work done by Dr Laura Andreae.

Figure 5.1: Developmental switch in vesicle cycling modes: (**a**) Schematic diagram showing the time line of the experimental protocol. Hippocampal neurons transfected with biosyn were first pre-incubated with strep488 (green) to quench the surface biosyn. The evoked and the spontaneous release were labeled with strep555 (red) following a high potassium treatment for 90s or left for 15 minutes in the presence of TTX and 0mM Ca^{2+} at 37°C respectively. (**b**) Graph showing a plot of the fluorescence intensities of the evoked and spontaneous release as a function of the days *in vitro*. n = 9-19 cells per condition/ time point. Values are shown as mean ± s.e.m.

5.3- Vesicle fusion is mainly spontaneous in immature neurons

The absence of evoked release early in development (4 DIV) was further confirmed using synaptophysin-phluorin (sypHy), a sensitive reporter of presynaptic vesicle cycling (Granseth et al., 2006).

In one set of experiments, 4 DIV and 14 DIV neurons expressing sypHy were incubated in the presence of bafilomycin for 15 minutes to allow spontaneous release to happen. Images were taken at 5min intervals to establish a time course for spontaneous release. Neurons were then depolarised with a high potassium solution (60 mM) in the continued presence of bafilomycin to release any activity-dependent vesicles that remain unmobilized. In both mature and immature neurons, most of the spontaneous pool was found to be released within 15 minutes. Whereas 14 DIV neurons showed a significant increase in sypHy florescence in response to a subsequent KCl challenge (Fig. 5.2 d,e), 4 DIV neurons did not respond to the stimulus (Fig. 5.2a,b).

In another set of experiments, neurons expressing sypHy were stimulated with 40 APs at 20 Hz in the absence of bafilomycin. This resulted in a transient increase in fluorescence in 14 DIV neurons (Fig. 5.2f) corresponding to the exocytosis and endocytosis of synaptic vesicles, but no response was observed in 4 DIV neurons (Fig. 5.2c). A similar stimulation paradigm showed robust Ca^{2+} transients along the axon, suggesting that Ca^{2+} influx did occur in young axons, but could mobilize vesicles (Data not shown; Andreae personal communication). Taken together, these experiments demonstrate that vesicle cycling early in development is entirely spontaneous.

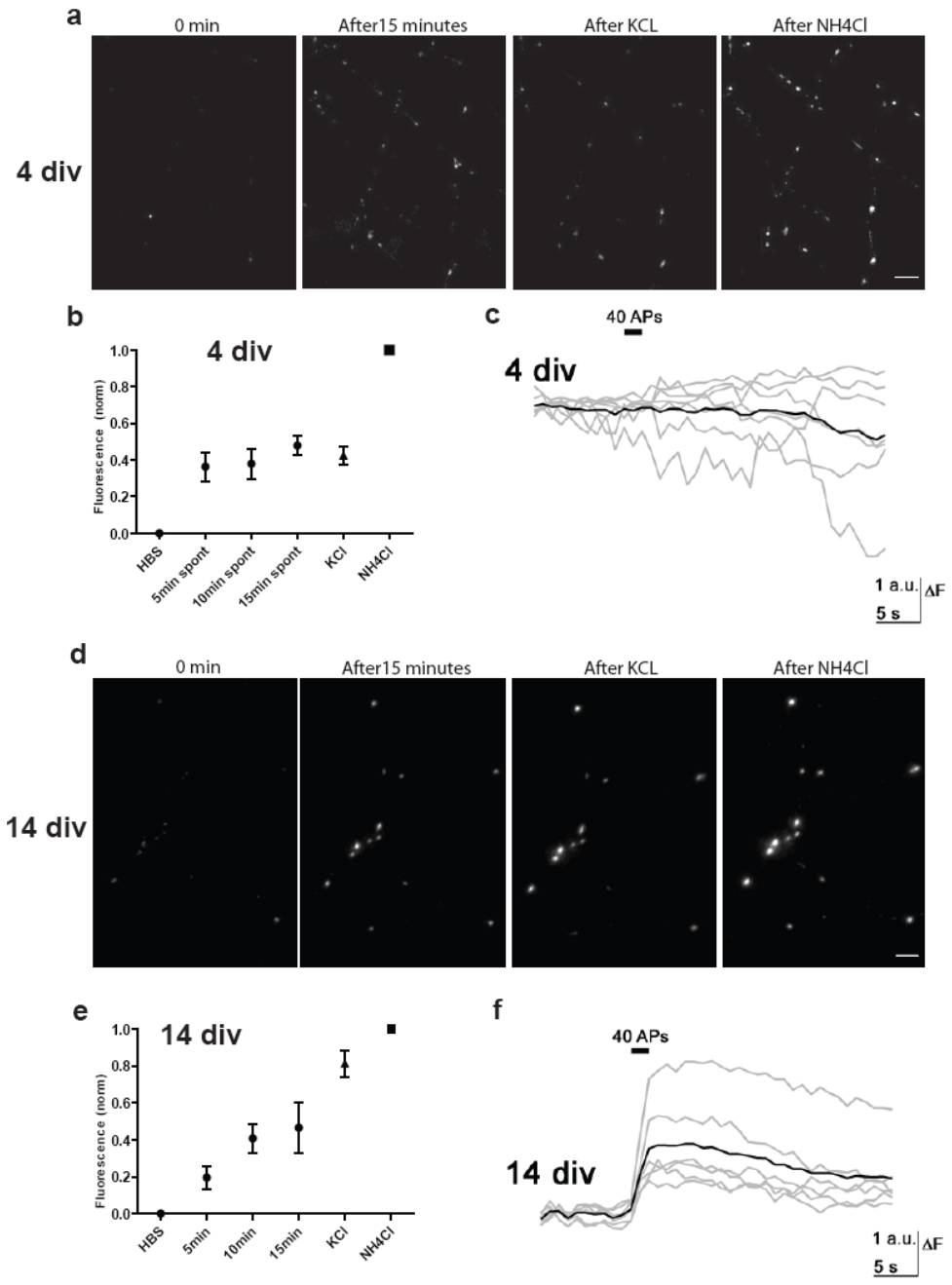


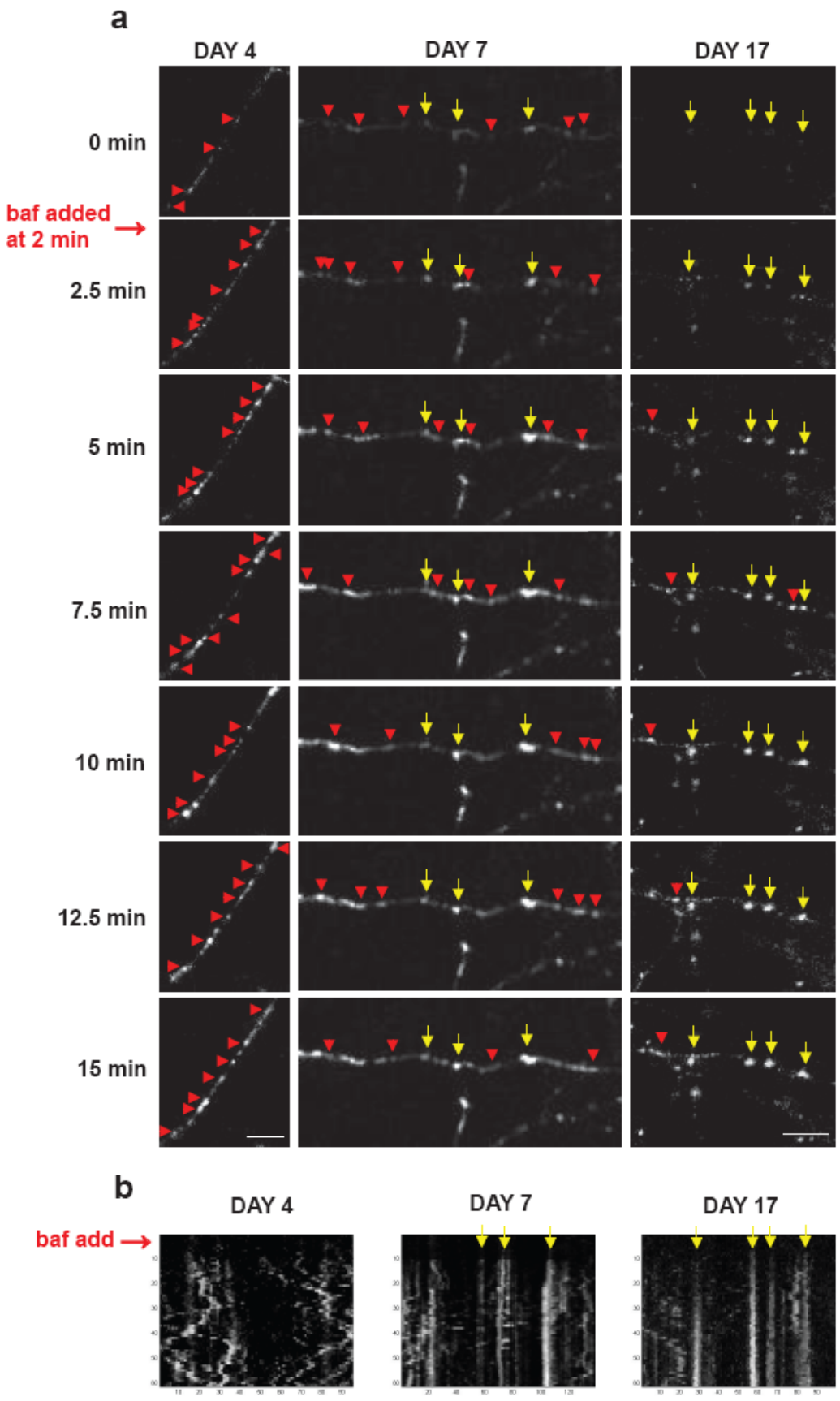
Figure 5.2: Immature neurons do not respond to activity. (**a**) Example images of 4 div neurons expressing sypHy in bafilomycin show no further increase in fluorescence following high KCl treatment. (**b**) Measurement of syphy fluorescence intensity during spontaneous release (●) and after high potassium (60mM) stimulation (▲), normalised to the total fluorescence obtained after NH_4Cl (■) for 4 DIV (n = 5). (**c**) Traces showing the absence of sypHy responses of a single synapse (gray) and the average response (black) to 40 action potentials at 20 Hz in 4 DIV neurons. (**d**) Example images of 14 DIV neurons expressing sypHy in bafilomycin showing an increase in fluorescence following high KCl treatment. (**e**) Measurement of syphy fluorescence intensity during spontaneous release (●) and after high potassium (60mM) stimulation (▲), normalised to the total fluorescence obtained after NH4Cl (■) for 14 DIV (n = 5). (**f**) Traces representing sypHy responses of a single synapse (gray) and the average response (black) to 40 AP at 20 Hz. Values are shown as mean ± s.e.m. Scale bar = 5µm.

5.4- Morphological analysis of vesicle cycling spontaneously during synapse development

Having established that mainly spontaneous release occurred early in development, time-lapse imaging was next used to monitor the trafficking of vesicles cycling spontaneously during synapse maturation. Neurons at different developmental stages (4, 7 and 17 DIV) expressing sypHy were imaged at 15 seconds intervals for 15 minutes at 37°C, in HBS lacking Ca^{2+} and in the presence of TTX. After 2 minutes of baseline imaging, bafilomycin was added into the medium to look for spontaneously recycled vesicles.

Different sizes of puncta, corresponding to clusters of vesicles, with diverse degrees of movements were found to recycle spontaneously during neuronal development (Fig. 5.3a, b). At 4 DIV, before synapse formation, puncta were generally of small size with a large spread of fluorescence intensities and highly motile (Fig. 5.3a, day 4, red arrowheads). They moved in an anterograde and retrograde direction, and coalesced into larger clusters or split into smaller ones. At 7 DIV, when synapses begin form, a mixture of large and small puncta is observed. The larger puncta, which correspond to mature presynaptic terminals, were in general brighter, stationary and represented the minority of clusters (Fig. 5.3a, day 7, yellow arrows), whereas the smaller puncta showed rapid movement along the axon and were highly over-represented (Fig. 5.3a, day 7, red arrows). At 17 DIV, when neurons have reached maturity and synapses are fully formed, a significantly higher number of large puncta (Fig. 5.3a, day 17, yellow arrow) and considerably fewer small ones were observed (Fig. 5.3a, day 17, red arrow). The stationary characteristic of these large puncta is nicely depicted in the kymographs (yellow arrows in Fig. 3 show large stationary puncta). Furthermore, kymographs of the different time points (4, 7 and 17 DIV) clearly show an increase in sypHy fluorescence of both large and small puncta after bafilomycin addition (Fig. 5.4b). These results demonstrate that motile and stationary puncta undergo spontaneous vesicle cycling in developing axons.

Figure 5.3: Morphological analysis of synaptic vesicle traffic during development. (**a**) Hippocampal neurons transfected with sypHy where time-lapsed for 15 minutes in the presence of TTX and 0mM Ca^{2+} at 37°C. Bafilomycin was added after 2 min. Yellow arrows indicate stable puncta and red arrowhead represent motile puncta. Note the increase in the puncta size as the neurons mature. Scale bar = 5 µm. (**b**) Kymographs showing both the movement of vesicles and the increase of sypHy fluorescence following addition of bafilomycin. The kymograph at DIV 4 clearly shows the absence of stable puncta as compared to 17 DIV.


a
DAY 4
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0 min
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5 min
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b
DAY 4
DAY 7
DAY 17
baf add

5.5- Vesicle cycling at the growth cone

Each growing axon possesses a growth cone that serves to navigate its way to target neurons. Growth cones comprise a central domain (or C domain) rich in microtubules and a peripheral domain (or P domain) containing actin, corresponding to filopodia and lamellipodia (Fig. 5.4 a) (Pfenninger, 2009). Having defined that in an isolated developing axon spontaneous vesicle cycling is the main form of vesicle fusion, we next examined if and how this process occurred in growth cones. Indeed, conflicting results have been reported regarding the specific location of vesicle cycling at the growth cone (Kraszewski et al., 1995; Diefenbach et al., 1999; Sabo and McAllister, 2003; Tojima et al., 2007). In order to obtain further insights into both the morphology and the dynamics of synaptic vesicle cycling at the growth cone, 4 DIV hippocampal neurons expressing both GFP and biosyn were first incubated for 30s in HBS containing strep647 (Fig. 5.4, b growth cone 1, blue) or strep488 (Fig. 5.4 b, growth cone 2, green) to quench the surface biosyn. Neurons were then left for 15 minutes at 37°C in HBS minus Ca^{2+}, in the presence of TTX and strep555 to label vesicles cycling spontaneously (Fig. 5.4 b, growth cone 1 and 2, red). Figure 5.4, clearly shows that spontaneous uptake of strep555 (red) happens either at the C domain (growth cone 1, yellow arrow) or behind the C domain (growth cone 2, white arrow) and that no labeling was apparent at the P domain of both growth cones.

Time-lapse imaging was next performed to study the dynamics of synaptic vesicles at the growth cone. 4 DIV hippocampal neurons expressing sypHy were imaged every 15s for 25 minutes. Bafilomycin was added after 5 minutes of baseline imaging. Bafilomycin is a specific proton pump blocker that prevents the re-acidification of vesicles after exocytosis resulting in a cumulative increase in sypHy fluorescence and it is that property that should aid the detection of vesicles that have recycled. If it is true that vesicle cycling happens at the filopodia of the growth cone, then a local increase in sypHy fluorescence should be obvious at the leading edge of the growth cone. Figure 5.5 shows three examples of growth cones treated as described above. Following addition of bafilomycin a significant increase in the average fluorescence of sypHy occurred at the C domain of the growth cone where most of spontaneous vesicle cycling happens. No obvious increase in sypHy fluorescent was seen at filopodia localized at the leading edge of the growth cone (see materials and methods for details on the approach used for analyzing the change in fluorescence in the C domain and the

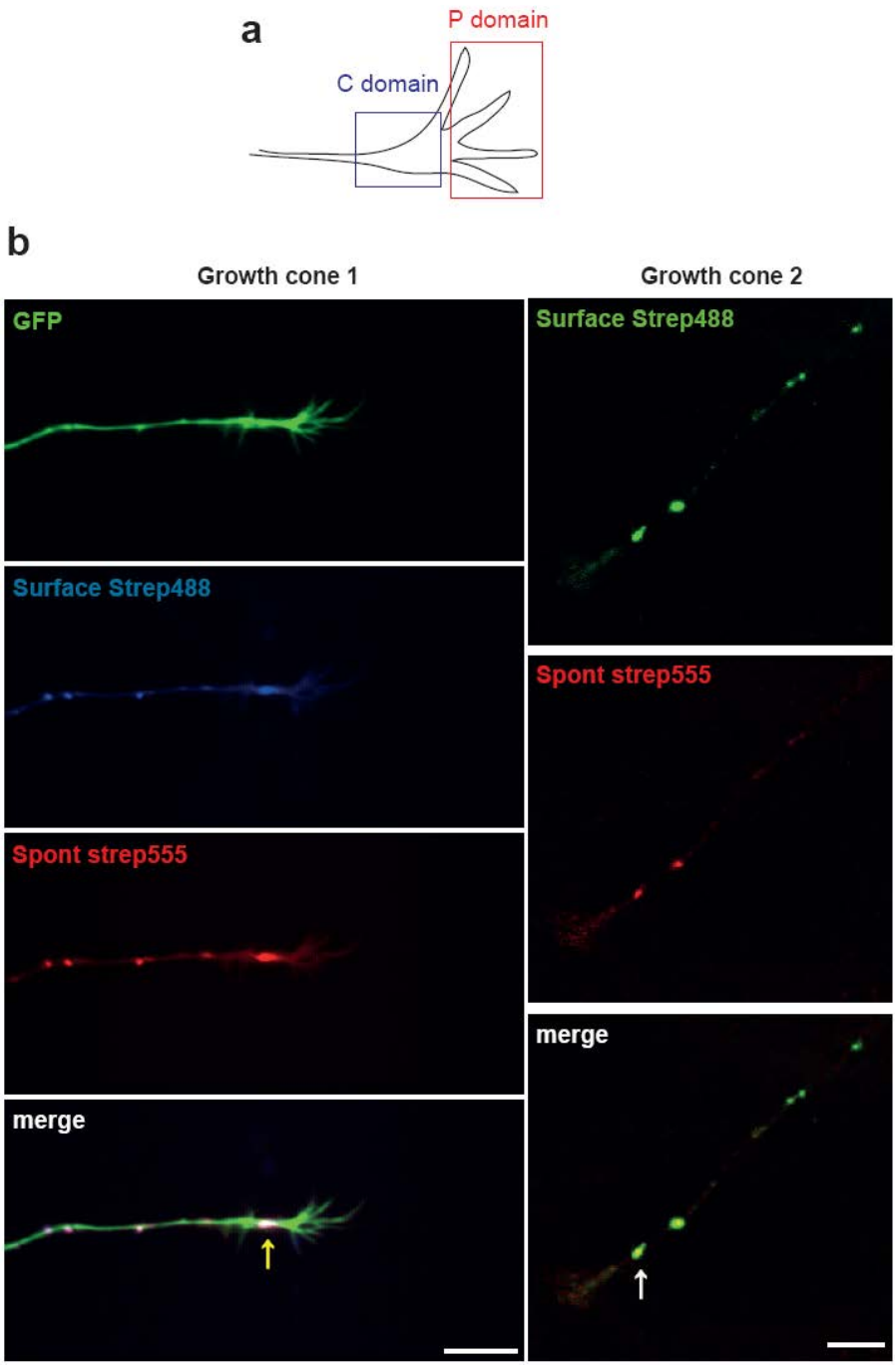

Figure 5.4: spontaneous vesicle cycling does not happen at the P domain. (**a**) Schematic diagram of a growth cone showing the localisation of the C (blue square) and the P domain (red square). (**b**) 4 DIV (growth cone 1) and 7 DIV (growth cone 2) hippocampal neurons were either co-transfected with GFP and biosyn (Growth cone 1) or biosyn alone (growth cone 2). Surface biosyn was stained with strep647 (blue: growth cone 1) or with strep488 (green: growth cone 2)for 30s. Neurons were then incubated for 15 min at 37°C in the presence of strep555, TTX and in the absence of Ca^{2+} to label spontaneous vesicle fusion. Neurons were fixed and imaged. The two growth cone examples show the presence of puncta behind the C domain (white arrows). Scale bar = 5µm.

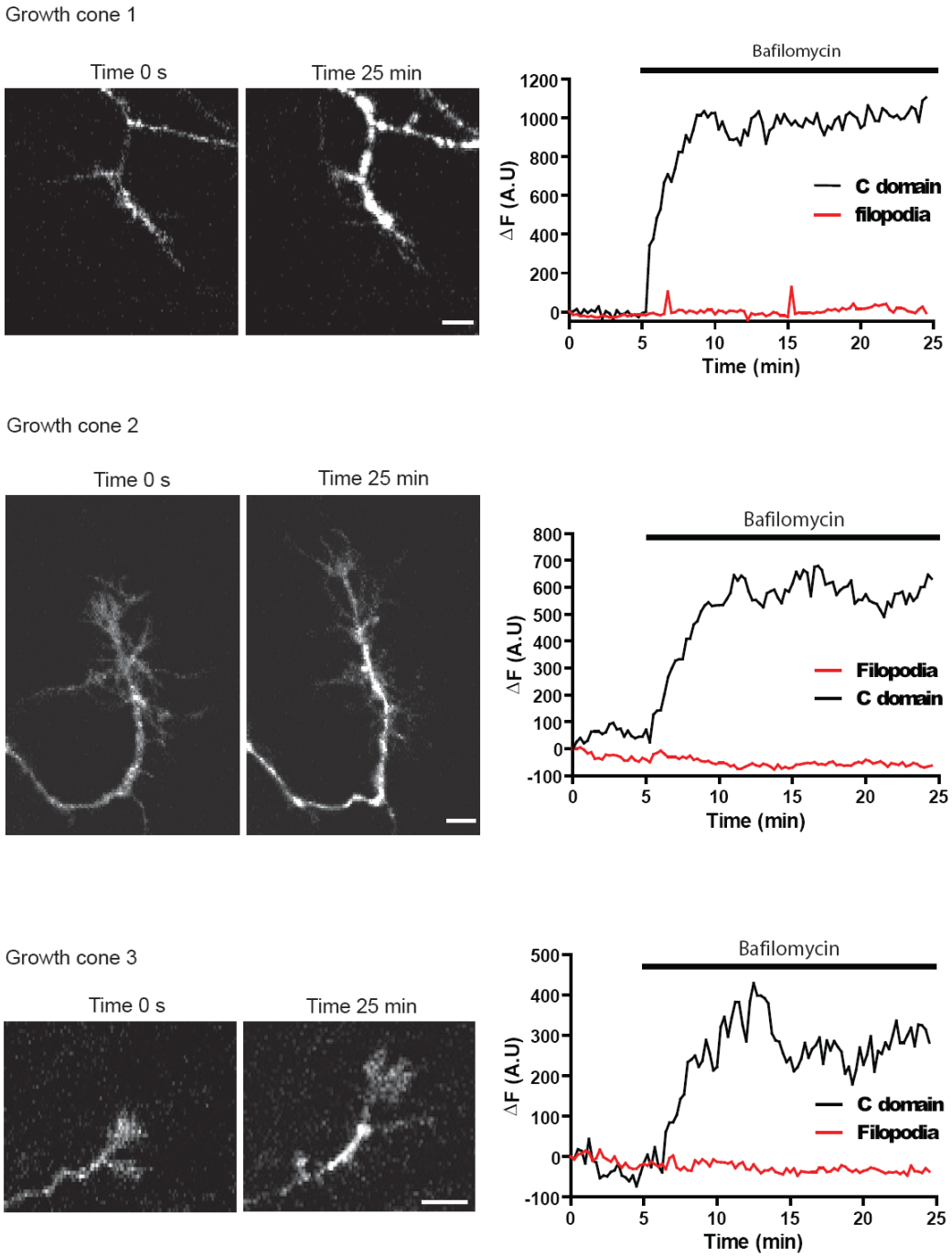

Figure 5.5: Spontaneous vesicle cycling at the growth cone. Three examples of growth cones expressing sypHy that were imaged every 15s for 25 min. Bafilomycin was added after 5 min of baseline imaging. Images on the left represent the growth cone before (at time = 0s) and after (at time = 25 min) bafilomycin addition. Note the elongation of the axon and the increase in fluorescence in C domain. Scale bar = 5µm. The graphs on the right represent the change in the average fluorescence intensity of sypHy over time. Following bafilomycin addition, a net increase in sypHy fluorescence is obtained in the C domain (black traces) while no change was found in the filopodia (red traces) of the three growth cones examples.

filopodia). However, highly motile puncta were found to move from the C-domain towards the highly dynamic filopodia (see Fig. 5.6) and were seen to move in anterograde and retrograde directions (green, white and yellow arrows). Sometimes puncta appeared *de novo* and then moved in an anterograde (white arrows) or in a retrograde (red arrow) direction. These puncta movements were independent of bafilomycin addition as similar motions were observed at the filopodia before addition of the drug, where faint vesicle clusters could also be observed.
Taken together, these results suggest that most of the spontaneous vesicle cycling happens at the C domain and that no vesicle cycling seem to occur at the P domain of the growth cone.

To further confirm these results, electron microscopy was performed on 4 DIV hippocampal neurons. Figure 5.7, show two examples of growth cones. View A and B represent the C and the P domain, respectively. While pleiomorphic vesicles were observed in the C domain, no vesicular structures were discerned in the P domain of both growth cones (Fig. 5.6, 1 and 2). Similarly, in figure 5.8, no vesicular structures were distinguished in the filopodia located at the leading edge of the growth cone (Fig. 5.8, 1) although multivesicular bodies as well as pleiomorphic vesicles were clearly visible in the C domain of the growth cone (Fig. 5.8, 2 and 3). One interesting observation is that filopodia arising from the axon shaft also show the presence of multivesicular bodies and pleiomorphic vesicles at the branching point (Fig. 5.8, B). Finally, vesicles were also observed at the tip of the filopodia that arose from the main axon (Fig. 5.8, A).

All together, these results show the presence of pleiomorphic vesicles in the C domain of the growth cone but no clearly defined vesicular structures in growth cone filopodia. In contrast, vesicles were observed in axonal filopodia. These preliminary results agree with some previous studies (Kraszewski et al., 1995) but contradict other studies showing that vesicle cycling happens at the leading edge of the growth cone (Sabo and McAllister, 2003). Further experiments are needed to further confirm these findings.

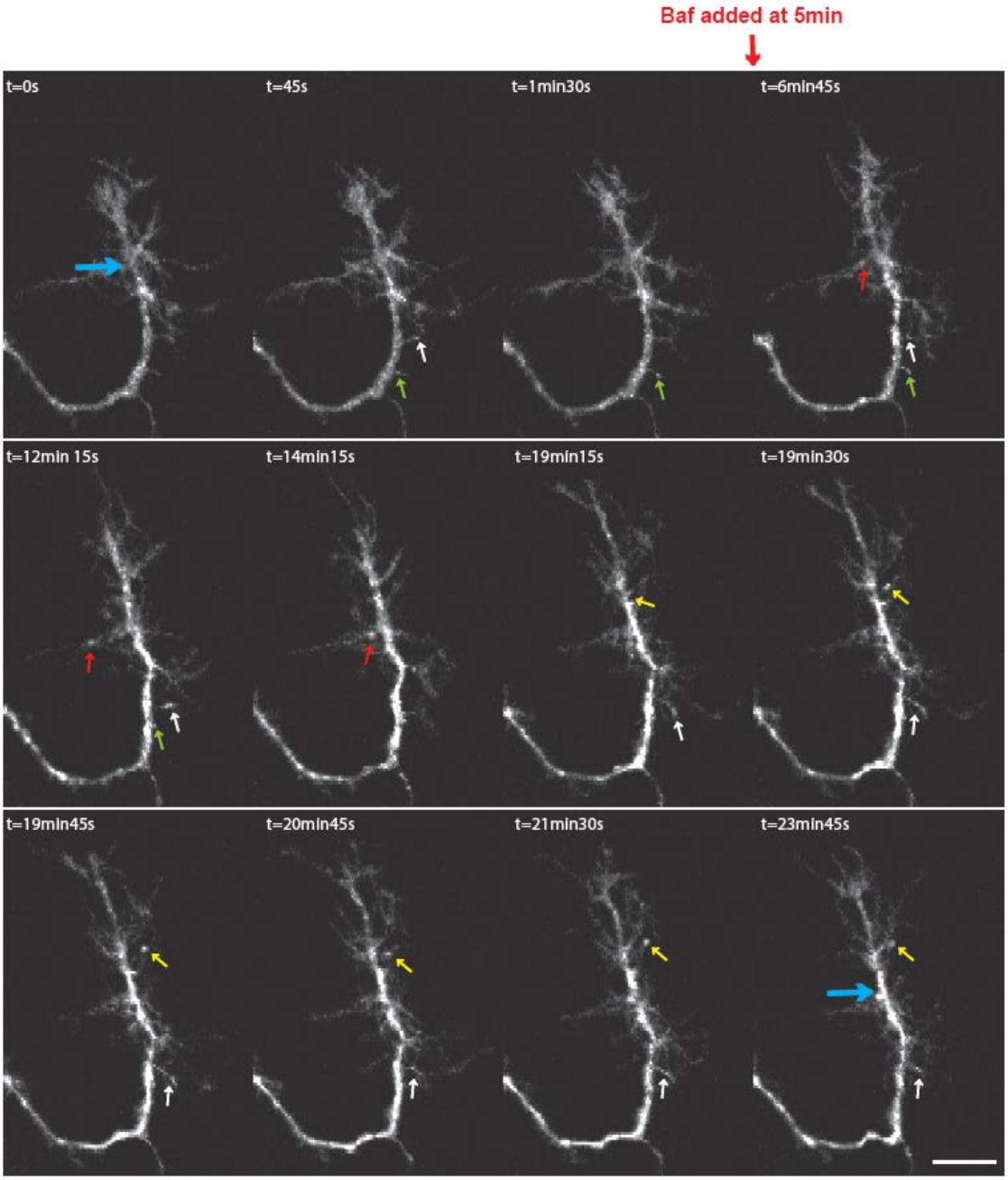

Figure 5.6: synaptic vesicles movements in a 4 DIV hippocampal growth cone. 4 DIV hippocampal neuron expressing sypHy was filmed for 25 min. Bafilomycine was added after 5 min of baseline imaging. The C domain shows a marked increase in sypHy fluorescence following bafilomycin addition. Arrows show the movement of vesicles into and out the growth cone filopodia (different colored arrows: refer to text). Scale bar = 10µm.

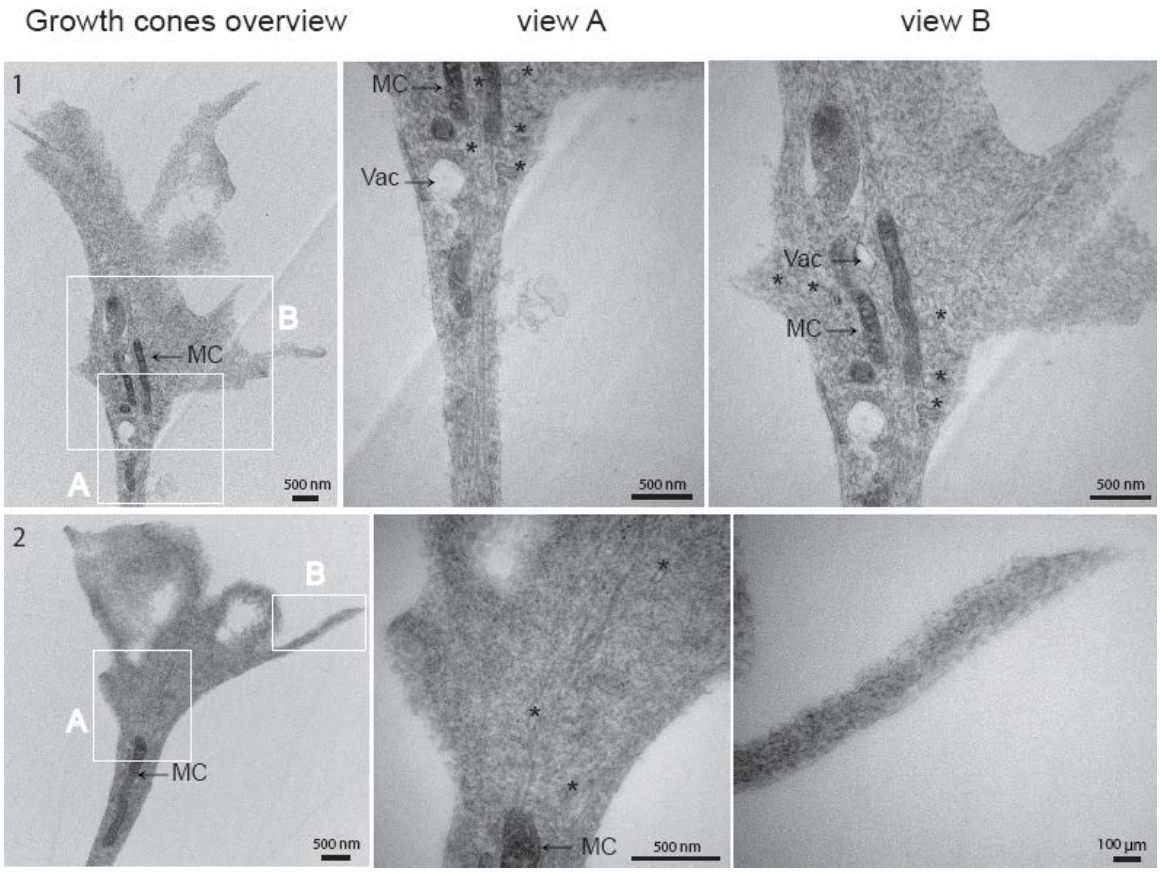

Figure 5.7: Ultrastructure of two 5 DIV hippocampal neuron growth cones. View A and B represent the C and P domain of the growth cone, respectively. Note the difference between a thin organelles-free filopodia (view B) and a thicker central region rich in organelles (view A). MC = mitochondria, Vac = vacuoles, * = pleiomorphic vesicles.

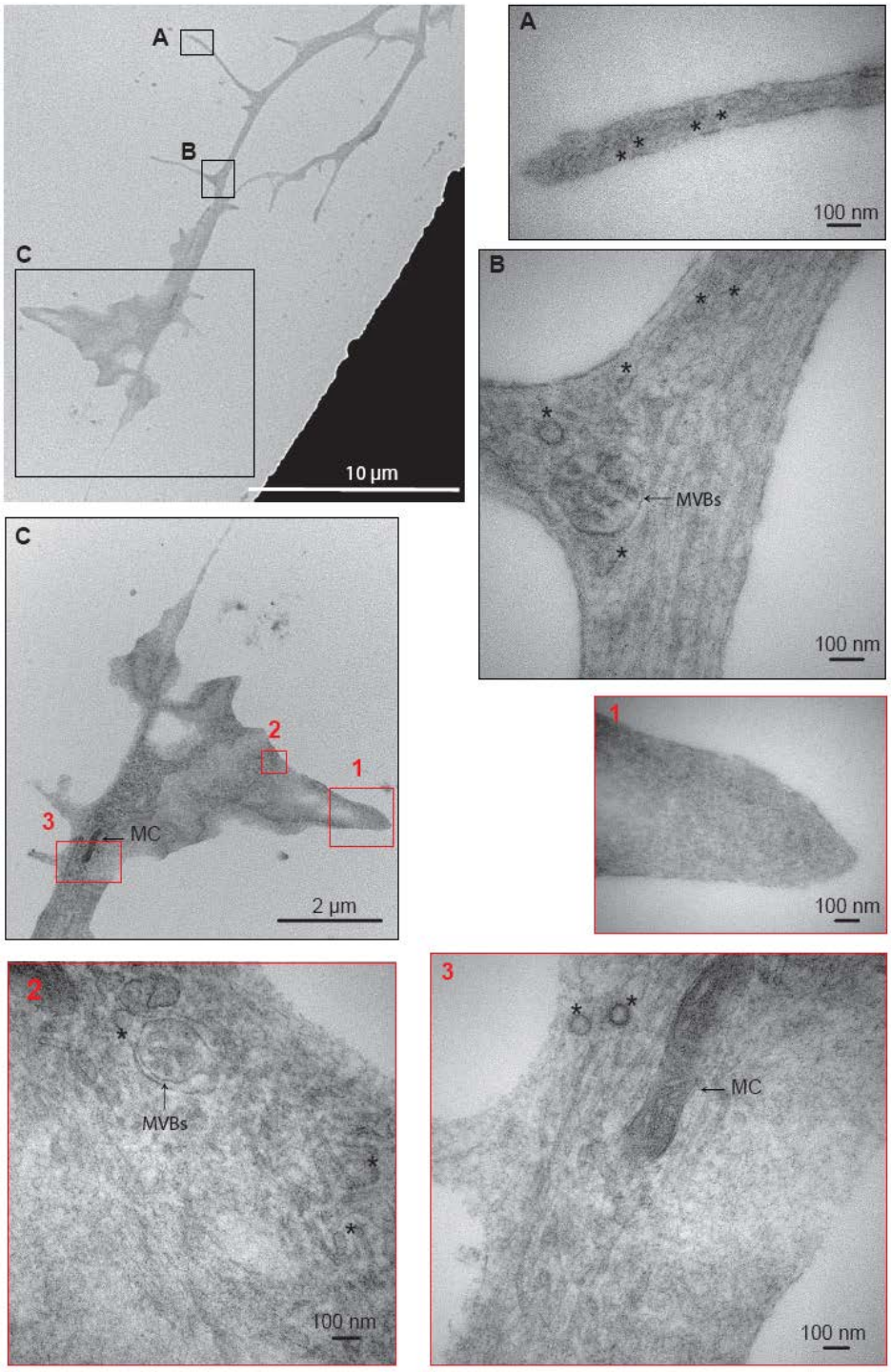

Figure 5.8: Ultrastructure of a 5 DIV growth cone and its axon: The first image on the left represents an overview of a growth cone and its axon. View A, B and C are higher magnification of the growth cone, axonal branching and axonal filopodia tip, respectively. View 1 is a higher magnification of a filopodia located at the leading edge of the growth cone A. View 2 and 3 represent different areas of the C domain of the growth cone A. Note the presence of pleiomorphic vesicles at the axonal filopodia tip (C) and at the axonal branching (B), and the absence of vesicular structures at the growth cone filopodia (1). MC = mitochondria, MVBs = multivesicular bodies, * = pleiomorphic vesicles.

5.5- Discussion

5.5.1- Summary of results

In summary, during neuronal development, young axons showed significant levels of vesicle cycling. More interestingly, a switch in the mode of synaptic vesicle fusion was found, where young neurons showed high levels of spontaneous cycling but no activity-dependent cycling, while mature neurons showed the opposite behavior: predominantly activity-dependent cycling, but hardly any spontaneous mobilization. In addition, we found that the size of the spontaneous pool in mature neurons is smaller than in immature neurons. However, both young and mature axons released the entire vesicle pool with similar kinetics and this release was complete within 15 minutes. In young axons, vesicle clusters were highly heterogeneous: small and large puncta were found along the axon and both were shown to undergo spontaneous fusion with the plasma membrane. The size of the puncta varied with the stage of development, with only small ones found in young neurons, which were also highly motile. In mature neurons, vesicle clusters were much larger and also stationary. Finally, at the growth cone, vesicle cycling occurred exclusively at the C domain and seemed to be absent from the P domain. Vesicular structures were also found at the axonal branching point and axonal filopodia.

5.5.2- Vesicle cycling is exclusively spontaneous in immature neurons

It has long been shown that vesicle cycling happens along a developing hippocampal axon before the formation of synaptic contacts (Matteoli et al., 1992; Kraszewski et al., 1995). This was first demonstrated by the internalization of an antibody directed against the luminal domain of synaptotagmin 1 (Syt1-Abs), an important synaptic vesicle protein (Matteoli et al., 1992). Based on this technology and the use of lipophilic dyes (FM dyes) (Betz and Bewick, 1992; Gaffield and Betz, 2006), spontaneous and activity-dependent recycling were described in immature neurons (Matteoli et al., 1992; Kraszewski et al., 1995; Zakharenko et al., 1999; Ahmari et al., 2000; Sabo and McAllister, 2003; Sabo et al., 2006). However, a higher level of spontaneous fusion was found to occur in young neurons when compared to mature synapses (Matteoli et al., 1992; Kraszewski et al., 1995; Coco et al., 1998). In this study, biosyn has permitted a detailed analysis of the spontaneous versus activity-dependent vesicle cycling during neuronal development. In chapter IV, it was shown that biosyn is a sensitive tool that allowed the labeling and characterization of the spontaneous pool. It was found that this

spontaneous pool was released within 15 minutes in mature neurons. Applying the same protocol on neurons at different developmental stages, the spontaneous pool in young neurons was found to be larger than in mature neurons after synapse formation. This finding is in accordance with previous results showing a down-regulation of spontaneous cycling as neurons progress in their maturation (Kraszewski et al., 1995; Coco et al., 1998). Furthermore, in the current work no activity-dependent cycling was found early in development. This result was further confirmed by the absence of sypHy changes in fluorescence following high potassium stimulation after the entire spontaneous pool of vesicles was released, and by the absence of responses to action potential stimulation. In previous studies, loading of FM dye into synaptic vesicles was achieved by stimulating the axon with high potassium. From this, the authors concluded that activity-dependent cycling did happen in immature axons and could be measured directly with FM dye loading (Sabo and McAllister, 2003; Sabo et al., 2006). However, our findings show that spontaneous vesicle cycling is prevalent in young neurons, which could result in FM staining during the long stimulation protocol used to stain vesicles with FM. This could result in erroneous conclusions where FM staining is taken to mean the presence of activity-dependent cycling, ignoring the ongoing high levels of spontaneous release. Indeed, during time-lapse imaging, the authors reported a significant loss of dye fluorescence that they attributed to spontaneous vesicle cycling (Sabo et al., 2006). Earlier studies using Syt1-Ab uptake in the presence or absence of a depolarizing stimulus have also reported that 2-4 DIV hippocampal neurons responded to depolarization (Kraszewski et al., 1995; Coco et al., 1998). However, due to the high level of spontaneous cycling we observed in young neurons, the activity dependent labeling they reported in these studies might have actually corresponded to spontaneous uptake occurring during the stimulation period. Indeed, a significant amount of Syt1-Abs uptake occurred in the control condition, in the absence of depolarization. It is important to note that, in some cases, activity-dependent vesicle cycling was clearly shown to occur in immature neurons (Dai and Peng, 1996; Zakharenko et al., 1999; Tojima et al., 2007), although these studies were performed in different species such as in Xenopus spinal neurons and in chick dorsal root ganglion cells. Species specific differences may account for these discrepancies.

5.5.3- Vesicle cycling occurs at the C-domain of the growth cone

Whether vesicle cycling occurs at the filopodia located at the leading edge of the growth cone remains a controversial issue (Kraszewski et al., 1995; Diefenbach et al., 1999;

Sabo and McAllister, 2003; Tojima et al., 2007). In this chapter, using biosyn, no spontaneous vesicle cycling was found to take place in growth cone filopodia. In addition, using sypHy combined with time-lapse confocal imaging to further explore spontaneous vesicle cycling in a live growth cone revealed the presence of highly motile vesicles in the filopodia that moved in retrograde and anterograde directions. The addition of bafilomycin did not show any increase in sypHy fluorescence in the filopodia, while sypHy present in the C-domain increased dramatically, providing further evidence that vesicle cycling does not seem to occur in filopodia. Similar results were found in previous studies using FM1-43 and an antibody directed against the luminal domain of synaptotagmin 1 where only the C domain in the growth cone was stained (Kraszewski et al., 1995; Diefenbach et al., 1999). This result was further confirmed when electron microscopy was performed. Indeed, vesicular structures were found in the growth cone C-domain but not in the growth cone filopodia (P-domain) (see Figure 5.7 and 5.8). Others reported similar ultrastructural observations in hippocampal neurons in culture (Mundigl et al., 1993; Kraszewski et al., 1995). By expressing VAMP2-EGFP or loading FM4-64 in growth cones of cortical neurons in culture, Sabo at al. also found the presence of highly motile clusters that moved rapidly and bidirectionally along growth cone filopodia (Sabo and McAllister, 2003). When treating these cultures with nocodazole, a drug that depolymerizes microtubules, Sabo *et al.* found a decrease in the movement of these clusters within the filopodia whereas the depolymerisation of actin with cytochalasin B did not have any affect (Sabo and McAllister, 2003). Similar results were obtained in the growth cone of a chick dorsal root ganglion (DRG) neuron loaded with FM1-43 where the transport of these clusters from the C-domain towards the P-domain was also found to be dependent on microtubules (Tojima et al., 2007). However, in contrast to our findings, Sabo *et al.* and Tojima *et al.* found that, using FM4-64 and a pH-sensitive version of Venus fused to VAMP2, the vesicles transported towards the P-domain recycled locally (Sabo and McAllister, 2003; Tojima et al., 2007).

It is important to define whether vesicles containing synaptic-vesicle proteins do recycle at the filopodia, as they have been implicated in playing an important role in axonal guidance signaling (Tojima et al., 2011). Further experiments are needed to further explore the presence or absence of local recycling at the growth cone filopodia.

5.5.4- Vesicle cycling during the maturation of the pre-synaptic terminal

5.5.4.1- Morphological analysis

In this chapter, time-lapse imaging of hippocampal neurons expressing sypHy has revealed the presence of two types of puncta. In young neurons, before synapse formation, puncta were small and highly motile. As shown in this study, activity-dependent cycling does not occur at that time of development; instead a high level of spontaneous cycling takes place. This suggests that the small puncta are only capable of spontaneous cycling. These highly motile puncta resemble the transport packets described by Ahmari et al. (Ahmari et al., 2000). They were found to contain presynaptic proteins including SV2, synaptotagmin 1, VAMP2, and voltage-dependent Ca^{2+} channel subunit (Kraszewski et al., 1995; Ahmari et al., 2000). Ultrastructural analyses of these transport packets revealed the presence of dense-core vesicles, pleiomorphic small vesicles and tubulovesicular structures that were suggested to be precursors of synaptic vesicles (Nakata et al., 1998; Ahmari et al., 2000). However, in another study, electron microscopy analysis of immuno-gold labeling of these puncta has revealed that they consisted of clusters of small clear vesicles similar to those found at mature presynaptic terminals (Kraszewski et al., 1995). Due to the highly dynamic nature of these puncta, one might wonder whether the composition of these clusters could vary depending on the developmental stage of the neuron.

In mature neurons, when synapses are fully formed, large and stationary puncta were observed. At that stage of development, activity dependent cycling becomes predominant and spontaneous pool of vesicles is smaller (Kraszewski et al., 1995; Coco et al., 1998; Ahmari et al., 2000; Fredj and Burrone, 2009). A previous study has shown that a clustering and stabilization of transport packets happened at sites of contact between neurons and resulted in the formation of large puncta followed by the appearance of activity-dependent release (Ahmari et al., 2000). Mozhayeva *et al.* have shown a correlation between the increase in the size of the presynaptic terminal accumulating vesicles and its function following postsynaptic contact (Mozhayeva et al., 2002). This opens the question of whether a postsynaptic contact is required for the maturation of the presynaptic apparatus and if not, how the mature presynaptic release apparatus is put in place.

5.5.4.2- Mechanisms

The general assumption made in the process of synapse formation is that a contact between a presynaptic axon and a postsynaptic partner is necessary for the maturation of a presynaptic button (Ahmari et al., 2000; Friedman et al., 2000; Jontes et al., 2000). Although this is likely the case for some important aspects of synapse function, in our lab (Andreae et al., 2012) it was found that the appearance of activity-dependent release does not require the contact with a postsynaptic dendrite. Indeed, a tight correlation was found between the size of the evoked pool of vesicle and the postsynaptic marker PSD95 at mature neurons (14 DIV), but no correlation was found at 10 DIV, suggesting that the appearance of activity-dependent cycling itself is independent from synapse formation and could be an intrinsic property of the neuron (Andreae et al., 2012).

By contrast, spontaneous vesicle cycling is present in both cases before and after a synapse is formed and might be important for the initiation and completion of this process. In chapter IV, it was shown that evoked and spontaneous release originates from two independent pools in mature neurons (see Chapter IV; (Sara et al., 2005)). Furthermore, it was recently found in our lab that the segregation of these vesicle pools occurs throughout neuronal development (Andreae et al., 2012). There is now mounting evidence that spontaneous neurotransmitter release (also known as `minis') can play an important role in synaptic function. For example, minis have been shown to influence neuronal firing in the small stella cells, where small conductances can have important effects on membrane voltage (Carter and Regehr, 2002). They have also been shown to play a role during homeostatic plasticity by regulating synaptic gain via the control of dendritic protein synthesis (Sutton et al., 2004; Sutton et al., 2006). They may also be implicated in structural remodeling of synapses. Indeed, blocking action potential by adding tetrodotoxin onto rat hippocampal slice cultures, resulted in both spine density and number remaining constant, whereas blocking both action potential firing and glutamate release (both evoked and spontaneous with botulinum toxins) resulted in a net loss of spines (McKinney et al., 1999). A better understanding of the role of spontaneous cycling early in development could thus give some hints on the mechanisms of synaptic plasticity in the adult. Further work is needed to investigate early spontaneous cycling and its regulation.

CHAPTER VI

Conclusion and perspective

6.1- Summary

In this study, I have developed a new genetically-encoded probe, biosyn, to investigate synaptic vesicle cycling in mature presynaptic terminals and throughout neuronal development. The generation of biosyn provided a novel way of labeling recycling synaptic vesicles by tagging a biotinylated form of VAMP2 with fluorescently labeled streptavidin. Using this approach in hippocampal neurons, I found: 1- two distinct pools of vesicles co-exist at the presynaptic terminal that recycle independently and with different kinetics. Whereas one pool is mobilized in response to neuronal activity the other fuses spontaneously with the plasma membrane. 2- the resting pool of vesicle, which cannot be mobilized by neuronal activity, is the source of spontaneous vesicle release. 3- a switch in the mode of synaptic vesicle fusion as neurons mature: from predominantly high levels of spontaneous cycling but no activity-dependent cycling in young neurons to predominantly activity-dependent cycling but hardly any spontaneous mobilization in mature neurons. 4- at the growth cone, vesicle cycling occur exclusively at the C domain and seemed to be absent from the P domain.

6.2. In vivo biotinylation: a promising tool for studying trafficking of membrane proteins

The use of techniques that allow the biotinylation of transmembrane proteins has grown in the past years and has shed light on the trafficking of surface antibodies, glutamate receptors (Predonzani et al., 2008) (Howarth et al., 2005) . In this thesis, I exploited this new development to study the trafficking of a specific synaptic vesicle protein: VAMP2. In so doing, I was able to indirectly track the vesicles that these proteins were associated with and therefore follow their fate. Two great advantages of this technique are (1) that there are no biotinylated proteins on the extracellular surface of neurons and (2) that the small globular protein streptavidin is easily washed away from the

extracellular medium. Together, these properties result in low background (non-specific) labeling, even after long periods of incubation with fluorescently-tagged streptavidin. These properties alone allowed us to detect spontaneous vesicle fusion events at individual synapses, with high signal-to-noise. Although biosyn has many advantages over other probes, it also has some drawbacks. The most obvious one is that it cannot report exocytosis directly as it happens. To solve this problem I have shown how combining sypHy with biosyn may provide an ideal combination for studying vesicle cycling at single synapses. Whereas sypHy can be used to measure the rates of exocytosis and endocytosis, biosyn can label different vesicle pools within the synapse and then provide a means to follow them over time. This approach exploits the advantages of both genetically-encoded probes and can therefore be used to better understand synapse function.

The biotinylation of transmembrane proteins to study their presence on the plasma membrane was greatly aided by the use of biotin ligases that allowed in vivo biotinylation of specific biotin acceptor domains, rather than rely on the addition of enzyme extracellularly. To achieve this, the biotin ligase enzyme, BirA, was localized to the secretory pathway by means of a signal secretion leader sequence. Following a similar logic, BirA could also be targeted to other compartments and provide a way of establishing whether proteins containing the BAP domain come into contact with this compartment. For example, this approach could be used to study the contact between cells at synaptic sites. BirA could be localized to the postsynaptic site of one neuron and a BAP-containing protein to the presynaptic membrane of another neuron. If the two membranes come together to form a synapse, the BAP domain will be biotinylated and could subsequently be detected by using fluorescent streptavidin. In this way, the process of synapse formation could be studied by exploiting the targeting of the BirA enzyme. This type of approach has been implemented in hippocampal neurons (Thyagarajan and Ting, 2010), which establishes a proof-of-principle that the idea may also work in neurons.

6.3- A separate pool for each mode of neurotransmitter release

Synapses sustain three modes of neurotransmitter release: synchronous, asynchronous and spontaneous release. Synchronous release is triggered by the arrival of an action

potential, resulting in an increase in intracellular Ca^{2+} at the presynaptic terminal. This results in the synchronous fusion of vesicles with the plasma membrane and the release of neurotransmitter into the synaptic cleft. The tight temporal coupling between Ca^{2+} influx and vesicle fusion results in neurotransmitter being released with sub-milliseconds delays, ensuring fast communication between neurons (Sabatini and Regehr, 1999). Asynchronous release, also known as "delayed release", follows synchronous release. It is driven by the residual, more global levels, of Ca^{2+} that occur in presynaptic terminals following the action potential. This form of release lasts for tens of milliseconds to seconds and is also Ca^{2+}-dependent, although to much lower levels of intracellular Ca^{2+} (Del Castillo and Katz, 1954; Goda and Stevens, 1994; Atluri and Regehr, 1998; Lu and Trussell, 2000). Kavalali and colleagues suggested that both synchronous and asynchronous release originates from the same pool of vesicles (Chung et al., 2010). However, the same group has recently shown that while VAMP2 drives fast synchronous release, VAMP4 specifically maintains asynchronous release (Raingo et al., 2012). This finding suggests that among the entire recycling pool of vesicles a subset of them are tagged with VAMP4 and are destined to support asynchronous release. Much like VAMP2, VAMP4 would make a stable SNARE complex with syntaxin-1 and SNAP-25 but it does not interact with complexin or synaptotagmin-1 suggesting that a different Ca2+ sensor may be required for asynchronous release. Finally, when synapses are at rest, they sustain a constitutive form of spontaneous release and I have shown that the resting pool of vesicle, which cannot be mobilized by neuronal activity, is responsible for this form of release. Although the molecular identity of this pool is still being debated, a recent study proposed that VAMP7 may be involved in spontaneous vesicle recycling (Hua et al., 2011b).

My results contradict this finding. Indeed, I did not find any role for VAMP7 in vesicle fusion of any sort (evoked or spontaneous) and concluded that VAMP7 was mainly endosomal marker that was not involved in membrane fusion events. Although it is tempting to attribute a different VAMP homologue to each form of release and VAMP7 was a prime candidate for spontaneous release due its insensitivity to tetanus toxin, our results do not support this idea. However, it is still possible that other VAMP molecules will, as yet unexplored, play a role. More importantly, Ramirez *et al.* have very recently identified another non-canonical SNARE protein Vps10p-tail-interactor 1a (Vti1a), previously thought to participate in the endosomal pathway, as being a molecular marker for vesicles fusing spontaneously (Ramirez et al., 2012). The most salient

feature of this study was that knocking down Vti1a in hippocampal neurons specifically inhibited spontaneous release, but left evoked release unperturbed. This is the first molecular alteration that leads to a selective decrease in spontaneous release and provides a unique opportunity to further characterize this unique pool of vesicles. A molecular difference between the two pools is further hinted at by a study showing the mobility of vesicles at rest. Those vesicles labeled spontaneously were significantly less dynamic than those labeled by activity, implying different vesicle pools may associate differently to the synaptic cytoskeleton. To test this, the authors used inhibitors of the motor protein myosin II, already implicated in the transport of vesicles, and found that evoked vesicles were more sensitive to the drug (their movement became restricted) than spontaneous vesicles. Although this is indicative of a difference between the two pools, the study did not directly measure the role of myosin II during stimulation, but only focused in synapses at rest (Peng et al., 2012). Overall, the molecular identity of spontaneous release has not been properly established. A significant advance in our understanding of the molecular composition of spontaneous vesicles would arise if a technique was developed that allowed their purification. Subsequent mass spectroscopy analysis would provide a detailed list of proteins on spontaneous vesicles, an approach already taken for the entire population of vesicles at the synapse (Takamori et al., 2006). One possibility to isolate pools of vesicles and establish their proteins identity would be to use biosyn together with a streptavidin linked to small magnetic particles. The magnetic biosyn-streptavidin complex could then be purified using a magnet, much like other antibody-based purification systems. One important caveat is that most available magnetic beads are too large (larger or equal in size to the diameter of synaptic vesicles) so cannot be used. However, a streptavidin linked to small iron-oxide nanoparticles (diameter of around 8-10 nm) is commercially available and could overcome this specific problem. The strength of the magnet required to pull down these vesicles will likely be the main issue with this approach, but if sufficient streptavidins are loaded into each vesicle, this drawback may also be overcome. Although clearly many hurdles remain the tools that are now available for labeling vesicles functionally may allow a full characterization of the molecules involved in the future.

6.4- Role of spontaneously fused vesicles in an immature neuron

While evoked neurotransmitter release has a well define function in ensuring fast communication between neurons, spontaneous release is less well understood. Although generally thought of as 'noise in the signal', spontaneous neurotransmitter release (also known as `minis') has been shown to play important roles in a number of synaptic events, such as influencing neuronal firing (Carter and Regehr, 2002; Sharma and Vijayaraghavan, 2003), regulating dendritic spine protein synthesis (Sutton et al., 2004; Sutton et al., 2006) and maintaining dendritic spines (McKinney et al., 1999). In the present study, a high level of only spontaneous vesicle cycling was found to occur in young neurons, in the absence of any axo-dendritic contact. This finding raises several questions: Do these spontaneous events release neurotransmitter? What triggers their fusion with the plasma membrane? What is the role of these spontaneous events? What are their molecular components?

There are two lines of evidence suggesting that spontaneous vesicle fusion occurring in an isolated axon might release neurotransmitter. First, using an antibody directed against the vesicular glutamate transporter VGlut, our lab found that it co-localised with vesicles that have been labelled with streptavidin following spontaneous vesicle fusion (Andreae and Burrone, unpublished observation). Other studies have also found VGlut1 present in vesicle found at the growth cone filopodia (Sabo and McAllister, 2003). Second, a more direct evidence for neurotransmitter release in an isolated axon was shown using electrophysiology (Young and Poo, 1983). As a probe for the release of acetylcholine (ACh), an outside-out patch of an embryonic muscle cell membrane expressing ACh receptors was used to measure release of ACh from growing axons. The authors found neurotransmitter release occurred all along the axon of Xenopus embryonic neurons, including at the growth cone. In fact, they found both spontaneous and evoked forms of release coexisted (Young and Poo, 1983). A similar approach could also be applied to growing axons of hippocampal neurons in culture. Future experiments could use cell lines that express high levels of AMPA receptors on the surface to perform similar out-side-out patch experiments. A more detailed use of this technique would combine ripped-off patches with spontaneous labelling of vesicles along an axon. By dragging a membrane patch along an axon, the levels of neurotransmitter release could be measured at release sites (hot-spots of labeled vesicles) and compared to areas of axons that are not labeled. This type of experiment

would be important to properly establish and quantify the levels of neurotransmitter release during early axon outgrowth.

Finally, who senses this release of neurotransmitter at a time when no synapses are yet present? Does neurotransmitter act in an autocrine fashion on receptors present on the same axon, or on neighbouring axons? Alternatively, could neurotransmitter be acting on neighbouring dendrites instead? Evidence for the role of glutamate receptor activation on young axons has come from imaging experiments on filopodia dynamics. Bath application of glutamate on young hippocampal neurons in culture (2 DIV) resulted in strong inhibition of the movement of axonal filopodia (Chang and De Camilli, 2001). More importantly, local application of glutamate onto short stretches of axon, also showed a similar phenotype on the perfused area, indicating glutamate was acting by locally activating glutamate receptors on the surface of axons themselves. Indeed, glutamate receptors (both ionotropic and metabotropic receptors) have been documented on immature axons and mature presynaptic terminals (Corlew et al., 2008; Pinheiro and Mulle, 2008), and have been shown to play a role in synapse function and plasticity (Corlew et al., 2008).

On the other hand, evidence for activation of dendritic glutamatergic receptors before synapse formation is less well documented. However, GABAergic signalling in developing neurons has been well characterised and shown to activate synchronous depolarizations known as giant depolarising potentials (Crepel et al., 2007). During these early periods, GABA receptor activation leads to membrane depolarisation through a non-synaptic form of GABA release that has been shown to activate postsynaptic neurons in the hippocampus. A similar role for glutamatergic signalling has not been observed. Perhaps the most striking observation for a possible role of glutamate early in development was described in a study that used uncaging of caged-glutamate near a dendrite in acute cortical brain slices from young mice (postnatal day 8-12). High frequency uncaging induced de novo growth of functional spines from the dendritic shaft (Kwon and Sabatini, 2011). Similar observations were also reported in hippocampal slices using ionophoretic application of glutamate to dendritic segments of a neuron (Richards et al., 2005). These studies suggest that neurotransmitter release from neighbouring axons might activate postsynaptic receptors on the dendritic shaft and act as a signal for either creating or guiding postsynaptic spines/filopodia to form a connection with an emerging presynaptic bouton. Our description of spontaneous forms

of release along an axon may provide the source of glutamate that result in postsynaptic activation of dendritic receptors and subsequent formation of synaptic contact. Future experiments will need to establish if postsynaptic dendritic events are observed before synapse formation. Unpublished data from the lab (Andreae and Burrone, unpublished observation) shows that spontaneous calcium rises are observed in the dendrites of immature neurons, which are driven by NMDA. This observation not only makes a case for non-synaptic neuronal communication, but may also provide a handle on the signals that drive synapse formation. To definitively confirm these results it will also be important to modulate spontaneous release from axons and show corresponding changes in dendritic calcium signals.

In this study, a developmental switch was found in the mode of release as neurons mature. The appearance of the evoked pool of vesicle coincides with a decrease in the spontaneous pool. Furthermore, it was found that the segregation of these vesicle pools occurs throughout neuronal development. Together these findings bring up further questions: are the vesicles that fuse spontaneously early in development the same as the ones that fuse in mature synapses? Does the switch in modes of release correspond to a molecular switch that gives rise to the evoked pool of vesicles as the neuron matures? If so what triggers that switch? Is it an extracellular signal or is it intrinsic to the neuron? Many of these questions still remain unanswered.

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